Title: Influence of hypoxic preconditioning in-vivo to 30 minutes knee surgery specific tourniquet application

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Influence of Hypoxic Preconditioning *in-Vivo* to 30 minutes Knee Surgery Specific Tourniquet Application

By

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A thesis submitted to the University of Bedfordshire in partial fulfilment of the requirements for the degree of Masters of Science by Research

October 2013

Abstract

Purpose: To establish whether a bout of hypoxic preconditioning (HPC) or ischemic preconditioning (IPC) would elicit a reduction in total knee replacement (TKR) surgery specific tourniquet mediated oxidative stress (OS) *in-vivo*. *Methods*: In an independent group design, 18 healthy men were exposed to 40 min of either: whole-body HPC (14.3% O_2), IPC (four bouts of 5 min ischemia and 5 min reperfusion) or rest (SHAM), 1 h prior to 30 min TKR specific limb ischemia and 2 h reperfusion. Systemic blood samples were taken at pre- and post-intervention, additionally blood and gastrocnemius samples were obtained at pre-, 15 min post- (15PoT) and 120 min post-tourniquet deflation. Systemic leukocytes and gastrocnemius tissue were analysed for the heat shock protein (Hsp72) and Heat shock protein 32 (Hsp32) gene transcript response (indicates severity of the cellular stress response), with the systemic plasma also assessed for OS markers (protein carbonyl and glutathione (reduced, oxidised, total, reduced/oxidised-ratio)). *Results*: A 1.93 and 1.97 fold reduction in gastrocnemius Hsp72 was noted in individuals exposed to HPC (p = 0.007) and IPC (p = 0.006) respectively, in comparison to SHAM at 15PoT. No significant differences were observed in gastrocnemius Hsp32, systemic Hsp72, Hsp32 or OS markers ($p > 0.05$) between groups. *Conclusions*: HPC and IPC provided cytoprotection to ischemic stressed gastrocnemius tissue as indicated by an attenuated cellular stress response to 30 min TKR specific limb ischemia.

Author's Declaration

I declare that the work presented in this thesis is entirely my own.

It has not been submitted for any degree or examination in any other University or educational institute.

James Barrington

30th October 2013

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Acknowledgments

I would like to thank my supervisors Dr Lee Taylor and Dr Bryna Chrismas. Their knowledge, expertise and academic guidance throughout this research process has been invaluable. I would also like to thank James Tuttle for his help and support with data collection and sample analysis. Additionally, the support given by the technical staff within the Sport and Exercise Science Laboratories was gratefully appreciated. I would also like to thank the participants who took part in the study for their patience and perseverance throughout the gruelling protocol. Also I would like to thank Mr Oliver Pearce for arranging surgeons to help with the study around their busy operating schedules. The delicate balancing act required to successfully achieve this would have undoubtedly caused a headache or two.

Personally, I would like to thank my parents for their love and encouragement (and delicious food) throughout my life. Without them I would not be the person I am today. Finally, I would like to thank my girlfriend Fiona Sharpe. Her love, support and unending belief in my ability has given me the confidence to pursue my aspirations.

Abbreviations

°C	degrees Celsius
µL	microlitre
4-HNE	4-hydroxy-nonenal
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AP1	activator protein 1
ATP	adenosine triphosphate
BH4	5,6,7,8-tetrahydrobiopterin
Ca^{2+}	calcium ion
CO	carbon monoxide
cm	centimetre
DNA	deoxyribonucleic acid
DTNB	5,5′-dithio-bis-2-nitrobenzoic acid
EC	endothelial cells
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-related kinase
ES	effect size
ETC	electron transport chain
G	gravitational force
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidised glutathione
H_2O_2	hydrogen peroxide
HPC	hypoxic preconditioning
HR	heart rate
hr	hour
HSF1	heat shock factor 1
Hsp	heat shock protein mRNA
HSP	heat shock protein
HXA	hypoxanthine
HYP	hypoxic preconditioning group
ICAM-1	intracellular adhesion molecule-1
IPC	ischemic preconditioning
IRI	ischemia reperfusion injury
JNK	c-Jun N-terminal kinases
K^+	potassium ion
kg	kilogram
L	litre
LMM	linear mixed model
LP	lipid peroxidation

MAPK	mitogen activated protein kinase
MDA	malondialdehyde
mg	milligram
min	minute
miRNA	microRNA
mK_{ATP}	mitochondrial potassium ATP
mL	millilitre
mmHG	millimetre of mercury
MMP	mitochondrial membrane potential
MnSOD	manganese superoxide dismutases
$mOsmols \cdot kgH_2O^{-1}$	miliosmalitymols per kilogram of water
mPTP	mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
ng	nanogram
nm	nanometre
NO	nitric oxide
NrF2	nuclear factor E2-related factor 2
O_2	oxygen molecule
$O_2^{\bullet-}$	superoxide
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PC	protein carbonyl
PKC	protein kinase C
RNA	ribonucleic acid
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
RT	reverse transcriptase
RT-PCR	real-time polymerase chain reaction
s	seconds
SHAM	sham-operated group
SNAP	s-nitroso-n-acteylpenicilleamine
TBARS	thiobarbituric acid reactive substances
TGH	total glutathione
TKR	total knee replacement
TLR	toll-like receptors
TNF-α	tumor necrosis factor-α
TOR	ischemic preconditioning group
XA	xanthine

XO	xanthine oxidase
XOR	xanthine oxireductase

Chapter 1: Introduction

1.1 General Introduction

Maintenance of homeostasis is paramount in the human body. This delicate balance is essential for efficient cellular function and survival of the body as a whole (Tortora and Grabowski, 1996). Stressful situations invoke disruption to the redox balance via a sharp increase in reactive oxygen species (ROS), diminishing endogenous antioxidants (such as the glutathione, MnSOD), thus instigating oxidative stress (Halliwell and Gutteridge, 2007). Although excess ROS induces oxidative stress and has been correlated with many disease states, low concentrations of ROS are essential for routine cellular signalling (Ray et al., 2012).

Tourniquets are widely used during total knee replacement (TKR) surgery to provide a bloodless field, improving visualisation of crucial structures and accelerating the surgical procedure (Smith and Hing, 2010; Estebe et al., 2011). Tourniquets have been used by medical practitioners since ancient Roman times (Klenerman, 1962), but there had been very little advancement in their design until the early 20th century when Harvey Cushing employed a pneumatic tourniquet system to limit bleeding during a craniotomy procedure (Fletcher and Healy, 1983). Nevertheless, there are negative features associated with the application of tourniquets, including, delayed wound healing, vascular injury and muscular damage (Estebe et al., 2011; Fitzgibbons et al., 2012), inviting controversy with regards to their use. In fact, delayed wound healing has been cited to defer patient discharge, incurring greater financial costs to the healthcare provider (Drew et al., 2007).

TKR surgery can last from (mean ± SD) 79.9 ± 12.7 min (Chang et al., 2012) to 145 ± 25 min (Horlocker et al., 2006) during which time a tourniquet is inflated. The interruption of blood supply mediated by the tourniquet, induces a hypoxic environment in the distal tissue (Clarke et al., 2001). Long periods of ischemia inhibit the regeneration of adenosine triphosphate (ATP) through aerobic sources, placing greater demands on the anaerobic glycolytic pathway (Ostman et al., 2004). The demand for ATP exceeds its replenishment leading to eventual adenine nucleotide degradation to produce the purine bases hypoxanthine and xanthine (Jennings and Reimer, 1991).

Upon reperfusion, the influx of oxygen initiates a rapid production of ROS via activated leukocytes, enzymatic degradation of purine bases and disruption of the vital mitochondrial electron transport chain (Granger et al., 1986; Carden and Granger, 2000; Murphy and Steenbergen, 2008). The large influx of ROS subsequently overwhelms the endogenous antioxidant defence systems stimulating oxidative stress (Adachi et al., 2006). Consequentially, triggering macromolecule damage to enzymatic structures, cellular lipid membranes and deoxyribonucleic acid (DNA) (Adachi et al., 2006; Ray et al., 2012; Brierley and Martin, 2013), thus inducing eventual cellular apoptosis and necrosis to the localised tissue (Jaeschke and Lemasters, 2003). This cascade of events has been dubbed ischemia reperfusion injury (IRI).

To diminish IRI, many agents and techniques have been applied, including administration of exogenous antioxidants such as, curcumin (Avci et al., 2012), caffeic acid (Ozyurt et al., 2006) and edaravone (Hori et al., 2013). Interestingly, the use of short cycles of ischemia and reperfusion primed the intended tissue and

bestowed protection for future ischemic insults (Murry et al., 1986). This phenomenon was termed ischemic preconditioning (IPC) and has been demonstrated to provide protection from IRI in both animal models (Mayr et al., 2004) and human participants (Koca et al., 2011). It is proposed that IPC operates in a biphasic modus through the activation of protein kinases, heat shock proteins (HSPs), *de novo* protein synthesis and stimulated transcription factors (Das and Das, 2008).

Various animal studies have used hypoxic stress as a preconditioning mediator to induce similar effects to IPC (Xi et al., 2002; Berger et al., 2010). It has also been demonstrated that consecutive whole-body hypoxic exposures attenuate the disruption to the redox balance caused by aerobic exercise in humans (Taylor et al., 2012). Indeed, hypoxic preconditioning (HPC) has been cited to function through similar mechanisms to IPC (Zuo et al., 2013). However, very few studies have investigated the effects of HPC on IRI in human skeletal muscle *in vivo*. Since HPC offers protection from redox balance disturbances (Taylor et al., 2012) and induces similar effects as IPC in animal models (Berger et al., 2010), it is therefore inferred that HPC would mitigate IRI in human skeletal muscle.

Considering that approximately 153,000 TKR operations were performed in England and Wales between 2008 and 2010 (Baker et al., 2013) costing on average £7500 (Dakin et al., 2012), the majority of the financial costs associated with TKR are incurred following the surgery via patient length of stay (Smith et al., 2008). Therefore, potential interventions to lessen tourniquet mediated tissue damage and delayed wound healing are crucial to reducing the length of stay and thus the financial burden upon health service providers.

1.2 Aims and Objectives

This thesis proposes to:

1) Quantify the time course for redox disturbances to the systemic and localised circulation via analysis of protein carbonyl (PC), reduced glutathione (GSH), oxidised glutathione (GSSG) and total glutathione (TGH), following hypoxic and ischemic preconditioning, in addition to immediately and 2 hrs succeeding tourniquet mediated ischemia.
2) Examine the time course for changes in Hsp72 and Hsp32 in localised skeletal muscle, in addition to localised and systemic leukocytes utilising the same time points as outlined in 1).
3) Evaluate the efficacy of both whole-body HPC and limb IPC based on the observed changes in 1) and 2) from TKR specific tourniquet application.

It was therefore hypothesised that:

- IPC and HPC would demonstrate a lower expression of Hsp72 in the localised muscle tissue (gastrocnemius) in comparison to control following TKR specific tourniquet application.
- Localised muscle Hsp32 would increase in HPC and IPC following tourniquet ischemia stress when compared to control.
- The systemic and localised circulatory redox markers (GSSG, PC) and stress protein expression (Hsp72 and Hsp32) would increase from the subsequent bout of TKR tourniquet application in the control condition in comparison to both HPC and IPC.

Chapter 2: Literature Review

This thesis is not a technical surgical paper, as such, will not contain novel operative procedures. The subsequent review will encompass a broad surmise of the major free radical species and the foremost antioxidant defence system, glutathione, in relation to the disturbed redox induced by the free radical entities. In addition, it will portray a logical progression of the events generated by the counter-intuitive phenomenon, IRI. Finally, the mechanisms responsible for potential preconditioning techniques will be explored, with particular interest focused upon the cytoprotective protein family, HSPs.

It should be noted that although this thesis concentrates on the negative effects of ROS, a large body of evidence is available suggesting the role free radicals play in hormesis (Nikolaidis et al., 2013) and normal cellular signalling, in both muscle (Powers et al., 2010a) and blood (Nikolaidis and Jamurtas, 2009).

The heart, kidney, liver and brain have been the most frequently studied within IRI due to the mortality rates associated with the failure of these organs. Therefore, the subsequent review has attempted to locate research concerning skeletal muscle, however research on other tissue has been utilised to demonstrate the point in question if skeletal muscle data is unobtainable.

2.1 Preoperative phase

TKR is an established treatment to alleviate the pain and discomfort associated with knee osteoarthritis as well as improving quality of life (Woolhead et al., 2005). Previous TKR studies have shown a mean length of stay of between 7.6 – 13.4 days (Rissanen et al., 1996; Smith et al., 2008; Jonas et al., 2013), which can be influenced through a variety of patient (social depravity, age, gender) and

hospital (peri-operative analgesia and care, recovery programmes) factors (Jonas et al., 2013). Reduced length of stay has been shown to be positively associated with increased patient satisfaction (Husted et al., 2008). To obtain patient insight with regards to the success of the TKR surgery, the Oxford Knee Score was developed, as potentially patients' perception of a satisfied outcome may differ from that of the surgeon (Dawson et al., 1998). Oxford Knee Score is specific to TKR surgery, with questions relating to pain and everyday movements rather than clinical and radiological data (Dawson et al., 1998).

There is vast pressure in the current economic climate to diminish National Health Service expenditure, particularly via cutting expenses (Dakin et al., 2012). Recently, research has attempted to establish early recovery programmes following TKR in an attempt to reduce hospital length of stay, with implementation demonstrating partial success (Smith et al., 2012). However, TKR often involves the routine application of a tourniquet to provide a bloodless field and improve visualisation of structures, thus reducing operative times (Abdelsalam and Eyres, 1995; Memtsoudis et al., 2010). Consequently, the occluded blood supply induces a hypoxic environment to the distal tissue (Clarke et al., 2001), increasing the risk of deleterious effects such as nerve palsy, metabolic disturbances, muscular injury and IRI (Fitzgibbons et al., 2012). The combination of these negative effects could perhaps explain the delayed discharge from hospitals noted in tourniquet versus non-tourniquet studies (Estebe et al., 2011).

Thus to maintain the advantages of tourniquet use (site structure clarity, reduced operative time) but attempt to minimise the associated deleterious effects, a

preconditioning intervention could be utilised. Therefore, it is necessary to establish homeostatic molecular events in order to comprehend the contrasting circumstances that occur through tourniquet mediated ischemia.

2.1.1 Redox Homeostasis

Prior to surgery in healthy individuals, redox homeostasis is maintained via an array of endogenous and exogenous antioxidant defence systems combating excessive pro-oxidant RONS (reactive oxygen and nitrogen species) (Halliwell and Gutteridge, 2007). At rest, the continuous production of RONS are associated with normal cellular metabolism (Valko et al., 2007). However, an increased production of pro-oxidants and concomitant failure of the anti-oxidant defence system initiates oxidative stress (Halliwell and Gutteridge, 2007). In this thesis, oxidative stress will be defined as; a disturbance to the redox balance in favour of ROS, leading to potential damage (Halliwell and Whiteman, 2004).

Below is a concise description of the major ROS produced by skeletal muscle tissue, the source of these radicals and their associated interactions with cellular components.

2.1.2 Free Radicals

Superoxide Anion

Superoxide ($O_2^{\bullet -}$) is produced through the transfer of an electron to the base-state oxygen atom, generally occurring in the “leaky” sites of the mitochondria electron

transport chain or from enzymatic reactions within the cell (Powers et al., 2010a). $O_2^{\bullet-}$ is considered a fairly weak ROS in comparison to others mentioned subsequently (Halliwell and Gutteridge, 2007), however, although the radical itself may not react directly, it can induce more potent species through its conversion (Figure 2.1) and has a relatively long half-life in comparison to other species (Halliwell, 1999; Powers and Jackson, 2008). Of note is the dismutation of $O_2^{\bullet-}$ through enzymatic (manganese superoxide dismutases) and spontaneous reactions providing a key source of hydrogen peroxide (H_2O_2) (Powers and Jackson, 2008).

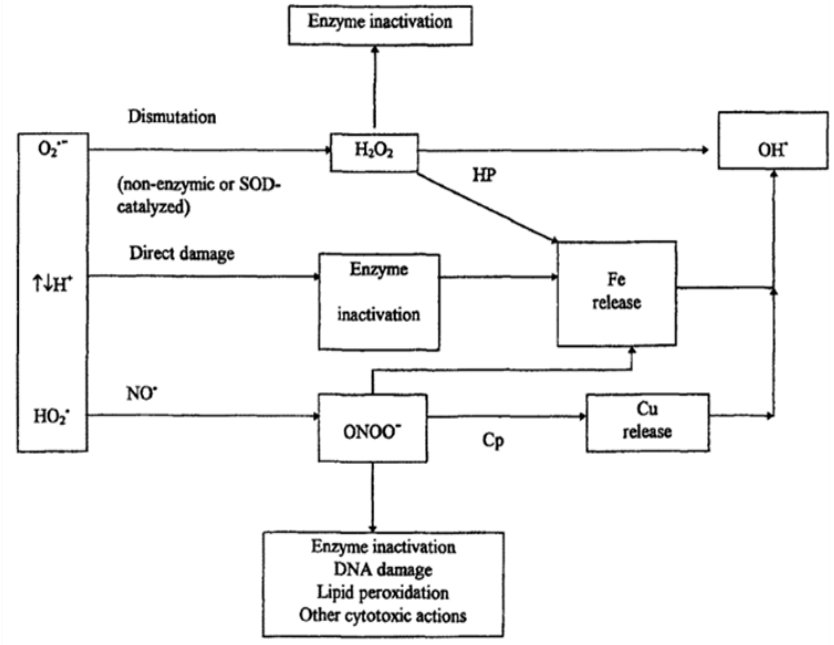

Figure 2.1: Mechanisms of the superoxide anion in initiating damage through its conversion. Abbreviations: $O_2^{\bullet-}$ - superoxide; H_2O_2 - hydrogen peroxide; $OH^{\bullet}$ - hydroxyl radical; $HO_2^{\bullet}$ - protonated superoxide anion; $NO^{\bullet}$ - nitric oxide; $ONOO^-$ - peroxynitrite; HP - heme proteins; Cp - caeruplasmin. Diagram taken from Halliwell (1999).

Hydroxyl Radical

Hydroxyl radicals ($OH^{\bullet}$) are highly reactive and due to this, are not membrane permeable, often reacting immediately with the surrounding environment. The majority of $OH^{\bullet}$ formed are transpired through Fenton chemistry of H_2O_2 catalysed by free heme (Halliwell, 1995). Indeed, research has demonstrated that the majority of the damage to DNA in cells treated with H_2O_2 is caused by $OH^{\bullet}$ (Spencer et al., 1995).

Hydrogen Peroxide

H_2O_2 is a relatively weak stable oxidising agent, however, it can permeate cell membranes and is readily produced, not just through dismutation of $O_2^{\bullet-}$ (Powers and Jackson, 2008) as mentioned previously, but also via other enzymatic reactions including xanthine oxidase (XO) (Sachdev and Davies, 2008). Although not very reactive, H_2O_2 is capable of inactivating certain enzymes directly, including the glycolytic enzyme GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Halliwell and Gutteridge, 2007) and is the main source of $OH^{\bullet}$ as mentioned previously.

Nitric Oxide (NO)

Nitric oxide (NO) is soluble in both water and lipids and has an array of biological roles including, vasodilation, neurotransmission and inhibition of platelet aggregation (Love, 1999). It is mainly synthesised through the conversion of L-arginine catalysed by the enzyme nitric oxide synthase (Powers and Jackson, 2008). Although not a reactive free radical, NO reacts readily with $O_2^{\bullet-}$ to form the reactive nitrogen specie, peroxynitrite ($ONOO^-$), which can lead to the production

of other damaging species (Brown and Borutaite, 2002). NO will react preferentially with other radicals and heme groups (Ferrer-Sueta and Radi, 2009).

Peroxynitrite

$ONOO^-$ is biologically generated through the reaction between $O_2^{\bullet-}$ and NO (Ferrer-Sueta and Radi, 2009). Although $ONOO^-$ is fairly stable, it is a strong oxidant, which reacts slowly with biological molecules, therefore largely influencing biological reactions within cells (Pacher et al., 2007). Upon pronation, ONOO- is converted to peroxynitrous acid which is extremely reactive and yields further oxidising and nitrating species (Arteel et al., 1999).

The production of RONS are imperative to correct regulation of wound healing (Sen, 2003). However, an accumulation of excess RONS leads to oxidative stress and eventual impairment in wound healing (Soneja et al., 2005). Therefore, it is inferred that oxidative stress produced via tourniquet inflation during TKR operations would negatively affect wound healing following surgery. Fortunately, the body has the ability to negate excessive pro-oxidants and maintain redox homeostasis via an antioxidant defence system.

2.1.3 Antioxidant defences

The body has a multitude of antioxidant defence systems to defend against cellular free radical disruption. Endogenous systems include the enzymes glutathione reductase, catalase and superoxide dismutase among others (Halliwell, 1999). In addition, exogenous sources that are gained through dietary intake such as vitamin C and E also supplement the antioxidant defence

(Halliwell, 1999). However, detail descriptions of these are not in the scope of this thesis. Accordingly, following the account above, the subsequent section will provide a thorough description of the antioxidant system glutathione as this particular marker was assessed during the experimental trial.

2.1.3.1 Glutathione

Glutathione is the most abundant non-protein thiol found in animal cells which is synthesised exclusively in the cytosol and is distributed throughout intracellular organelles including the nucleus, mitochondria and the endoplasmic reticulum (Mari et al., 2013). Intracellular glutathione is predominantly found as GSH, accounting for around 99% of the TGH found in the majority of human tissue (Halliwell and Gutteridge, 2007). Degradation of GSH to its oxidised form (GSSG) occurs exclusively in extracellular spaces, thus involving membrane transporters to deliver glutathione to different organelles or extracellular spaces (Ballatori et al., 2009). The biosynthesis of glutathione involves glutamate, cysteine and glycine through the enzymatic action of glutamate cysteine ligase and glutathione synthase (Figure 2.2) (Maher, 2005). GSH can neutralise H_2O_2 either through enzymatic (glutathione peroxidase) or non-enzymatic reactions generating GSSG, which is then available to be recycled back into GSH to aid further in cellular protection (Figure 2.2) (Maher, 2005; Halliwell and Gutteridge, 2007).

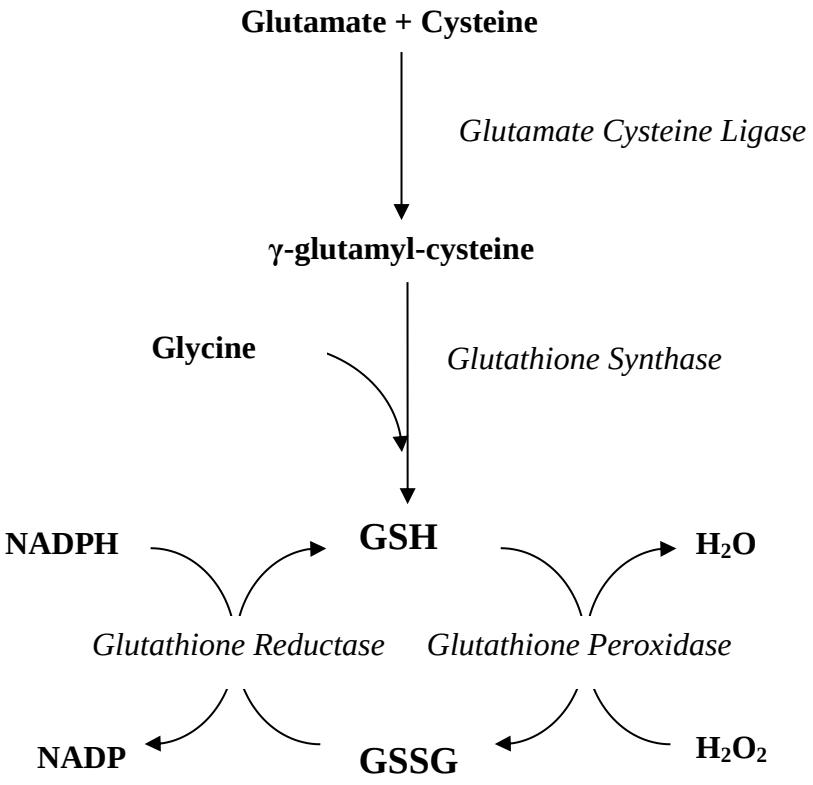

Figure 2.2: Glutathione synthesis and metabolism pathways. Abbreviations: GSH – reduced glutathione; GSSG - oxidised glutathione; NADPH – reduced nicotinamide adenine dinucleotide phosphate; NADP – nicotinamide adenine dinucleotide phosphate; H_2O_2 – hypdrogen peroxide; H_2O – water. Adapted from Maher (2005).

Glutathione is often measured in whole blood samples due to the low concentration of TGH found in plasma (0.5%) and vast levels in erythrocytes (99.5%) (Serru et al., 2001). It has been previously used in varying protocols to assess oxidative stress in hypoxia (Taylor et al., 2012), dehydration (Hillman et al., 2011) and ageing (Jones et al., 2002) among others.

The attenuation of GSH has been associated with delayed wound healing (Rasik and Shukla, 2000), therefore, monitoring concentrations of this tripeptide would provide useful when assessing tourniquet mediated redox disturbances.

Multiple methods are available to analyse glutathione samples from human blood and muscle tissue (Rahman et al., 2006), the choice made regarding the presented thesis is detailed in section 3.9.

2.1.4 Cellular Damage

If the antioxidant defence systems are overwhelmed oxidative stress will ensue (Halliwell and Gutteridge, 2007). Excessive cellular damage of lipids, proteins and DNA following oxidative stress is common in delayed wound healing (Soneja et al., 2005). Below is a general description of the mechanisms that produce lipid peroxidation, protein oxidation and DNA damage following oxidative stress.

2.1.4.1 Lipid Peroxidation

Free radicals induce structural changes to cellular membranes, affecting their functional capacity, thus allowing more direct free radical attacks upon intracellular proteins (Halliwell and Chirico, 1993). In addition, lipid peroxidation (LP) increases membrane permeability to ions, giving rise to intracellular increases of Ca^{2+} inducing disruptions to cellular metabolism (Halliwell and Chirico, 1993; Niki, 2008).

Radical mediated peroxidation mechanisms involve the removal of protons from polyunsaturated fatty acids to produce a lipid radical (Gueraud et al., 2010). This initiates a propagation reaction via lipid radical oxidation, which in turn reacts

with a fresh lipid, invariably creating another lipid radical and the unstable lipid hydroperoxyde, inevitably continuing the chain reaction (Gardner, 1989; Gueraud et al., 2010). Termination of the reaction only occurs when the creation of non-radical and non-propagating species transpires (Gueraud et al., 2010).

LP is thought to be associated with various human disease states such as Alzheimer's disease (Pratico and Sung, 2004), atherosclerosis (Minuz et al., 2006) and IRI (Adachi et al., 2006). Measuring LP is usually performed analysing the stable products of lipid hydroperoxyde, such as malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE) or isoprostanes (Powers et al., 2010b). MDA for example is commonly assessed via the use of thiobarbituric acid reactive species (TBARS) assay kits, however, most of the TBARS found in human samples appear not to be related to LP or MDA, therefore use of high performance liquid chromatography is recommended as a preferred method (Halliwell and Whiteman, 2004).

2.1.4.2 Protein Oxidation

ROS undergo numerous reactions (electron transfer, hydrogen abstraction, re-arrangement) with protein peptides during oxidative stress, ultimately disrupting the functionality of the protein structure (Hawkins and Davies, 2001). The majority of free radical attacks are focused upon the peptide side-chains and the protein back-bone, forming a multitude of radicals due to the variability of potential sites upon both of these protein fragments (Hawkins and Davies, 2001). The predominant product of protein oxidation are carbon-centred radicals, which are well known precursors to PCs (Sibrian-Vazquez et al., 2010). The most

frequently used and reliable technique for assessing protein oxidation is via the reaction between 2,4-dinitrophenylhydrazine and PC for spectophotometry analysis, based on methods by Levine et al. (1994). Indeed, PC quantification is particularly pertinent within this thesis as protein oxidation stimulates the activation of HSPs and the heat shock response (Noble et al., 2008) (detailed further in section 2.3.2.1).

2.1.4.3 DNA Damage

DNA damage is considered the most serious of ROS induced alterations as DNA is merely copied, provoking mutations into the base sequence of replicated nucleic acids (Poulsen, 2005). The majority of DNA damage is caused by ROS and it is estimated that 2×10^4 damaging events occur in every human cell every day (Barzilai and Yamamoto, 2004). This ROS mediated damage occurs due to oxidising of nucleic bases, splitting DNA cross-links and breaking single/double DNA strands (Barnes and Lindahl, 2004). Fortunately, the body operates effective systems in repairing DNA modifications through base-excision and nucleotide-excision repair pathways (Brierley and Martin, 2013). DNA alteration is considered to be a pathophysiological factor in the development of cancer (Poulsen, 2005) and is often detected via oxidised by-products utilising high performance liquid chromatography and mass spectrometry techniques (Weimann et al., 2001).

Increased protein damage is associated with delayed wound healing and is speculated, in part, to be produced via oxidative stress (Moseley et al., 2004).

Therefore, alleviation of tourniquet mediated oxidative stress following TKR surgery may help to reduce wound healing durations.

2.2 Peri-Operative

The induction of oxidative stress through increased radical production and the concomitant failure in antioxidant defences invariably leads to cellular structural degradation. The subsequent description will outline the molecular events that occur during tourniquet inflation in the peri-operative phase of TKR surgery, eventually leading to the developing stages of oxidative stress and associated cellular damage.

2.2.1 Ischemia

Tissue distal to the tourniquet becomes ischemic and hypoxic (Clarke et al., 2001), consequentially disturbing aerobic metabolism, invariably placing greater demands upon anaerobic sources (Grace, 1994). Oxygen is an essential fuel source for cellular metabolism, replenishing ATP concentrations via the electron transport chain (ETC) in the mitochondria (Tortora and Grabowski, 1996). However, during ischemia, glycolysis becomes the main source of ATP re-synthesis, but this process in turn, also increases the ratio of nicotinamide adenine dinucleotide phosphate (NADP)/reduced NADP (NADPH) in addition to lowering the pH, consequentially slowing the glycolytic process via inhibition of GAPDH (Jennings and Reimer, 1991). As ATP re-synthesis slows beyond cellular usage, adenosine diphosphate (ADP) concentrations rise, allowing two of these molecules to be converted into ATP and adenosine monophosphate (AMP), where the latter undergoes eventual degradation to hypoxanthine (HXA) if O_2 is not

restored (Jennings and Reimer, 1991). Under normal homeostatic situations, metabolites would be removed by the circulation, but during blood flow occlusion concentrations are allowed to accumulate (Grace, 1994). In addition, the hypoxic conditions also up-regulate the enzyme xanthine oxidoreductase (XOR) which catalyses HXA into xanthine (XA) and XA into uric acid in the presence of oxygen (Hassoun et al., 1998). Limb ischemia can last for up to (mean ± SD) 145 ± 25 min during TKR surgery (Horlocker et al., 2006). This prolonged blood occlusion can lead to significant cell necrosis and tissue damage, therefore restoration of blood flow is paramount (Lefer and Lefer, 1996).

Paradoxically, ischemia itself appears not to induce as severe damage in comparison to the re-introduction of oxygen after a bout of blood flow-occlusion (Parks and Granger, 1986a). This illogical phenomenon and subsequent tissue damage has been named IRI and has a very complex pathophysiology (Lefer and Lefer, 1996).

2.2.2 Reperfusion

An appropriate beginning is with the many cellular regions in which oxidative stress can be derived. Once surgery is completed and the tourniquet has been deflated, the re-occurrence of blood flow to the hypoxic tissue leads to a rapid induction of free radicals which has been cited as a major factor of oxidative stress leading to IRI (Grace, 1994). Outlined below are the major sources of free radicals which lead to the disruption of the redox balance and consequential cellular damage, and eventual delay in wound healing following IRI.

2.2.2.1 Mitochondrial Derived Free Radicals

The ischemic environment is hypothesised to disrupt the mitochondrial ETC; this disruption leads to inefficient electron transfer and increased ROS production upon reperfusion (Murphy and Steenbergen, 2008). Indeed, inhibition of the ETC at complex I during ischemia has been demonstrated to diminish IRI in cardiac tissue (Lesnefsky et al., 2004). Three extensively studied factors regulate mitochondrial ROS production; mitochondrial membrane potential (MMP), Ca^{2+} concentrations and NO availability (Zhang and Gutterman, 2007). High MMP promotes ROS generation via slowing of the ETC and prolonging the ubisemiquinone radical immediate at complex III, known to be a considerable site of $O_2^{\bullet-}$ production (Zhang and Gutterman, 2007).

Mitochondria play a key role in Ca^{2+} homeostasis, providing a transient location for Ca^{2+} storage, elevating inter-mitochondrial membrane Ca^{2+} concentrations (Bernardi, 1999). Ca^{2+} stimulates ROS generation through activation of the tricarboxylic acid cycle, thus enhancing the work rate of the mitochondrion and augmenting ROS output (Brookes et al., 2004). Ca^{2+} can also initiate NO production, leading to inhibition of complex IV, again enhancing the radical stimulating intermediate, ubisemiquinone (Cleeter et al., 1994). The ineffective electron transfer stimulates the production of the free radical $O_2^{\bullet-}$, damaging mitochondrial proteins and further exacerbating the disruption upon the ETC (Baines, 2009). The polarity of $O_2^{\bullet-}$ makes it difficult for diffusion across the mitochondria membrane, with the mitochondrial permeability transition pore (mPTP) serving as the only conduit for its transport (Han et al., 2003). The most

important mechanism for intermembrane transport of $O_2^{\bullet-}$ is via its conversion into the uncharged H_2O_2, which can easily diffuse across membranes into the cytosol (Zhang and Gutterman, 2007).

The role of mitochondrial derived ROS is well established in the pathophysiology of sustained ischemia and reperfusion (Lesnefsky et al., 2004; Baines, 2009), thus would contribute to macromolecule damage and redox disturbances observed in tourniquet application (Koca et al., 2011).

2.2.2.2 Xanthine Oxireductase

The accumulation of HXA during the ischemic bout allows for its conversion via XOR once molecular oxygen is restored and has been implemented as a key contributor of ROS production during ischemia-reperfusion (Granger et al., 1986). XOR has two inter-convertible forms; XO and xanthine dehydrogenase both of which are capable of converting HXA and XA, only differing due to the former able to catalyse exclusively via reducing oxygen (Figure 2.3) (Berry and Hare, 2004).

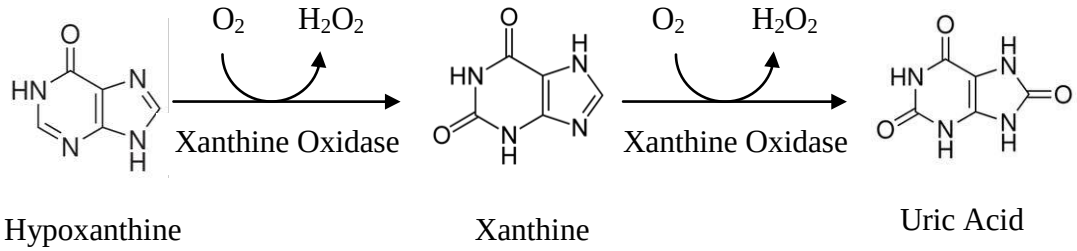

Figure 2.3: Diagram representing the degradation of hypoxanthine following the re-introduction of oxygen. Abbreviations: O_2 – oxygen molecule; H_2O_2 – hydrogen peroxide. Adapted from Berry & Hare (2004).

The conversion of XOR to XO is initiated in hypoxic conditions (George and Struthers, 2009) and is the main component for XOR derived ROS via generation of by-products H_2O_2 and $O_2^{\bullet-}$ (Berry and Hare, 2004). In mammals, the highest proportion of XOR is observed in the small intestine and the liver (Parks and Granger, 1986b), although in humans the majority of XOR is an inactive state (Berry and Hare, 2004). It has also been reported that it exists solely in the cystoplasm of cells (Ichikawa et al., 1992), although this is under some debate (Frederiks and Vreeling-Sindelarova, 2002).

Several lines of evidence have implicated XO in the pathophysiology of IRI. Anoxic aortic endothelial cells demonstrated large bursts of ROS after ischemia-reperfusion, and were linked to the activity of XO (Zweier et al., 1994). Indeed, XO derived by-products have also been linked to cardiac dysfunction in rat hearts after ischemia-reperfusion, which was reduced following treatment with the ROS scavenger allopurinol (Brown et al., 1988). Furthermore, implementation of XO inhibitors abolished free radical creation in rat brain during a bout of ischemia-reperfusion (Phillis et al., 1994). These findings were also supported by another XO inhibitor (BOF-4272) which abolished lipid membrane peroxidisation in hepatocytes in an *in vitro* model (Kakita et al., 2002). However, there is controversy to whether XO actually contributes to the ischemia reperfusion radical production. Lindsay et al. (1990) performed 5 hrs of ischemia upon *ex-vivo* canine gracilis muscle and measured small increases in IRI precursors HXA and XA, in addition to a minor increase in XO activity between 5 and 15 min after initial reperfusion. Nevertheless, there were no apparent rises in uric acid which would be the expected by-product from this enzymatic reaction. Surprisingly, this

article did fail to assess any markers of tissue damage which may have provided a clear link to whether the low conversion of HXA/XA to uric acid corresponded to similar levels of tissue injury.

As tourniquet inflation has been attributed to inducing IRI (Estebe et al., 2011) and XO is considered to contribute to IRI (Carden and Granger, 2000), it therefore could be speculated that XO participates in tourniquet mediated ROS generation.

2.2.2.3 Leukocytes

The significance of XO derived ROS in IRI has been disputed with some authors favouring the role of leukocytes as the major source of ischemia-reperfusion mediated oxidative stress. Under normal circumstances, synthesis of NO is produced by endothelial nitric oxide synthase (eNOS) utilising molecular oxygen and L-arginine in the presence of the essential co-factor 5,6,7,8-tetrahydrobiopterin (BH4) (Perkins et al., 2012). However, BH4 is easily oxidised to dihydrobiopterin if oxidant radicals overwhelm key intracellular antioxidant defence mechanisms such as glutathione or vitamin E (Crabtree et al., 2008). The reduction in BH4 concentrations cause eNOS to switch from NO production to the $O_2^{\bullet-}$ radical, thus furthering oxidation of BH4 (Schmidt and Alp, 2007; Crabtree et al., 2008). A reduction in NO release has been cited frequently in relation to IRI (Lefer and Lefer, 1996; Carden and Granger, 2000; Khanna et al., 2005; Perkins et al., 2012), and appears to be related to the abrupt release of $O_2^{\bullet-}$ upon reperfusion inhibiting NO synthesis (Ma et al., 1993). Moreover, as mentioned in section 2.1.2, the increased availability of $O_2^{\bullet-}$ provides NO the ability to react and

produce the potent radical $ONOO^-$ accumulating to the exacerbation of oxidative stress (Crabtree et al., 2008).

Leukocytes are recruited to the ischemic site via cytokines, chemokines and cellular selectins expressed on the endothelial cells (ECs) (Jaeschke, 2003). Leukocytes free-flowing in the microcirculation pass the ECs and respond to inflammation mediated selectins, progressively slowing in a process known as leukocyte rolling (Kansas, 1996). P-selectin expressed on ECs has been implicated as the dominate receptor for leukocyte rolling (Carden and Granger, 2000), which is found within intracellular Weibel-Palade bodies and is rapidly exteriorised to the cell surface membrane within minutes of agonist (histamine, thrombin, H_2O_2) stimulation (Kansas, 1996; Lefer and Lefer, 1996). P-selectin glycoprotein ligand-1 on the surface of leukocytes is the principle ligand for P-selectin and results in intergrin activation which links leukocyte rolling with fixed cellular adhesion (Langer and Chavakis, 2009). ß2 intergrin (CD11b/CD18) present in leukocytes have also been implemented as the principle molecule for firm adhesion of leukocytes to the EC membrane via the membrane's counter-receptor, intracellular adhesion molecule-1 (ICAM-1) (Lefer and Lefer, 1996). Once bound, the activated leukocytes release proteases which are capable of EC membrane degradation, in addition to release of H_2O_2 and $O_2^{\bullet-}$ inducing oxidative stress (Carden and Granger, 2000). Furthermore, bound leukocytes infiltrate the tissue through the endothelial barrier resulting in greater endothelial damage and edema formation (Nedrebo et al., 2003).

Strong evidence implicates the role of leukocytes in IRI. Investigation into mice deficient in CD11/CD18, P-selectin or ICAM-1 demonstrated complete protection from mesenteric artery EC injury after a 45 min bout of ischemia-reperfusion (Banda et al., 1997). These results were corroborated by Connolly et al. (1996) who found a 3.7 fold reduction in infarct size from 45 min ischemia followed by 22 hrs reperfusion on the cerebral artery in ICAM-$1^{(-/-)}$ mice compared with ICAM-$1^{(+/+)}$ control mice. Furthermore, canines administered with a CD18 intergrin neutralising antibody demonstrated a diminution of myocardial infract size in comparison to control after ischemia-reperfusion (Duilio et al., 2001). As previously mentioned, NO has been intrinsically linked with IRI. Indeed, inhibition of endogenous NO synthesis increased vascular leukocyte rolling in humans (Hossain et al., 2012). In addition, administration of NO (via NO donor S-nitroso-N-acteylpenicilleamine (SNAP)) has also shown to be effective in diminishing leukocyte rolling and adhesion (Dal Secco et al., 2006). However, the administration of SNAP has been shown to aggravate IRI through greater free radical generation (Zhang et al., 2003) demonstrating the difficulty of exogenous NO donor administration in attempting to prevent IRI.

These results clearly depict an integral role for leukocyte, mitochondrion and XOR in contributing to greater free radical production (Figure 2.4) and ensuing oxidative stress experienced during IRI. Hence, the oxidants produced through XOR during initial re-oxygenation suppress NO release and augmented $O_2^{\bullet-}$ production initiating leukocyte rolling and increased oxidant production respectively, thus inevitably disrupting the redox balance. As redox homeostasis is paramount in avoiding cellular injury (Zweier and Talukder, 2006), diminishing

the associated redox balance disturbances during tourniquet inflation/deflation would minimise the chance of wound complications associated with oxidative stress (Soneja et al., 2005; Estebe et al., 2011).

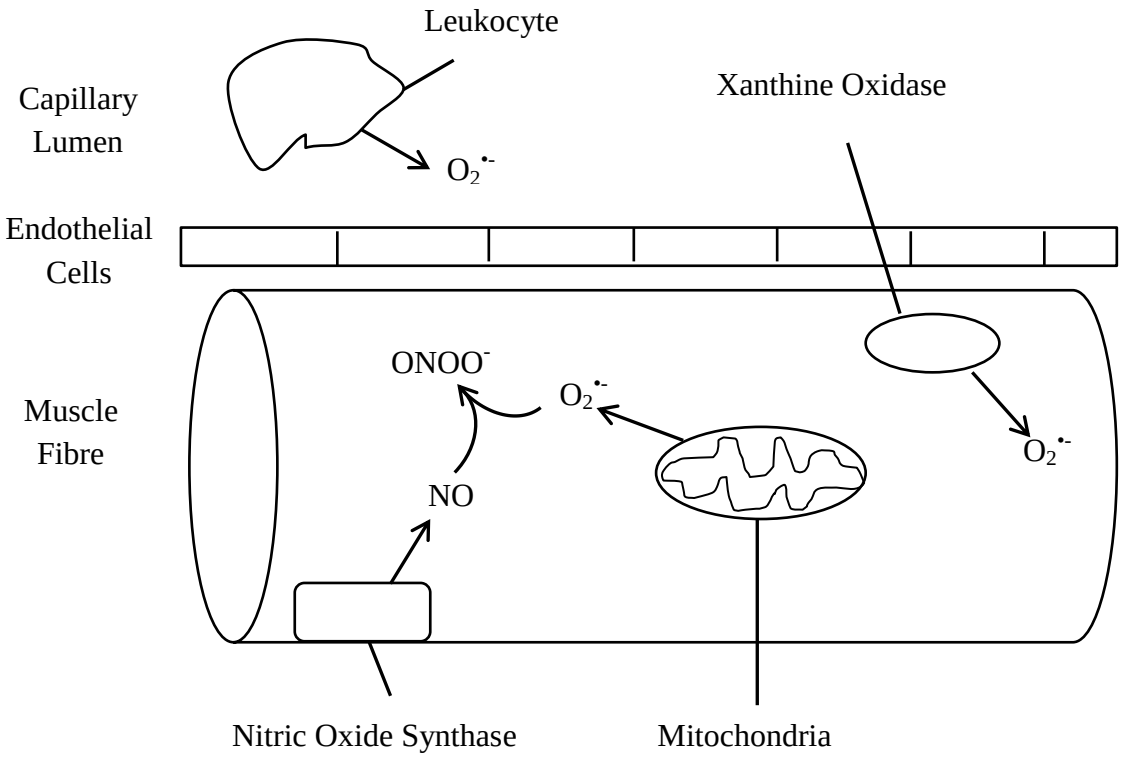

Figure 2.4: Illustration of potential reactive oxygen species (ROS) production during ischemia-reperfusion. Abbreviations: $O_2^{\bullet-}$ - superoxide anion; NO – nitric oxide; $ONOO^-$ - peroxynitrite. Adapted from Powers et al. (2010).

2.2.3 Glutathione and Ischemia-Reperfusion

As mentioned in section 2.1.3.1, glutathione is a key endogenous thiol protein intended to neutralise free radical molecules (Halliwell and Gutteridge, 2007). The free radical production induced via ischemia-reperfusion has been measured frequently in IRI studies to assess the redox disruption. Tissue homogenates

(muscle, liver) are more commonly measured in rodent models. Puntel et al. (2013) noted a reduction in GSH/GSSG ratio following 3 hrs of ischemia and 2 hrs of reperfusion in rat gastrocnemius muscle. A reduction in GSH concentrations alone have also been reported post 4 hrs ischemia and 2 hrs reperfusion in rat gastrocnemius muscle (Avci et al., 2012). During aorta-biformal bypass surgery on elective patients, GSH concentrations were noted to significantly decrease 24 hrs after initial reperfusion from ischemia (median ± range; 113 ± 21) in comparison to preoperative levels (Westman et al., 2006). Surprisingly, the ratio of GSH/TGH was not significantly different from preoperative levels, as augmented oxidative stress would be expected to create greater concentrations of GSSG, diminishing the GSH/TGH ratio (Halliwell and Gutteridge, 2007). Although this may be due to extracellular exportation of GSSG (Ballatori et al., 2009).

The literature collectively presents the disruption caused to the localised tissue concentrations of GSH via ischemia-reperfusion. However, controversy exists in relations to glutathione disruption in blood sampling. A significant increase in GSSG was noted in systemic blood taken from the antecubital region at 3 and 10 min in reperfusion after TKR surgery (Garcia-de-la-Asuncion et al., 2012). Interestingly, whole blood samples obtained from the operated knee also displayed an increase in GSSG but at much greater concentrations. This disparity between observed GSSG/GSH at localised and systemic sites has also been noted by Karg et al. (1997). The authors demonstrated a significant increase in the whole blood GSSG/GSH ratio at 5 min reperfusion in the operated leg when compared to samples obtained from the arm. Finally, whole blood sampled at the

site of ischemia in knee surgery patients (femoral vein) acquiesced a significant change in whole blood GSH and GSSG in comparison to sampling from a systemic source (antecubital vein) (Mathru et al., 1996). Research from Garcia-de-la-Asuncion et al. (2012), Karg et al. (1997) and Mathru et al. (1996) indicate that the site of blood sampling can affect the yielded concentrations of glutathione from a bout of ischemia-reperfusion. Therefore, GSH/GSSG concentrations will provide a useful insight into redox changes induced via the hypoxic intervention following TKR specific tourniquet application.

2.2.4 Ischemia-Reperfusion and Macromolecule Damage

The large oxidative burst initiated by reperfusion has a detrimental effect upon localised tissue damage once the antioxidant defence is overwhelmed. Increased levels of lipid peroxidation (4-HNE) were observed after 3 hrs of ischemia and only 5 min of reperfusion in murine hind limb (Adachi et al., 2006). Hori et al. (2013) found increased concentrations of MDA at 24 hrs reperfusion following 1.5 hrs of ischemia in both rat gastrocnemius and anterior tibialis. Furthermore, increased levels of TBARS were observed at 10 min reperfusion in a clinical population undergoing lower-leg extremity surgery (mean ± SD; 78.7 ± 13.3 min of tourniquet inflation) when compared with baseline figures (Van et al., 2008). However, as previously mentioned, TBARS are not specifically generated via LP (Halliwell and Whiteman, 2004), therefore the results should be interpreted with caution. Interestingly, ischemia alone induces lipid peroxidation, and is exacerbated further upon reperfusion (Paradies et al., 1999). Greater lipid peroxidation was also noted in hepatic (Lee et al., 2000) and brain tissue (Sakamoto et al., 1991) post ischemia-reperfusion.

The deteriorated integrity of the cellular membrane from the reperfusion associated oxidative attack leads to intracellular protein degradation (Halliwell and Chirico, 1993). Similar to lipid peroxidation, the literature displays decisive evidence for the role of ischemia-reperfusion in increasing protein oxidation. PC concentrations were elevated in rat gastrocnemius muscle following 4 hrs of ischemia and 2 hrs reperfusion in comparison to a SHAM condition (Avci et al., 2012). These results were collaborated by Ozyurt et al. (2006) who noted a rise of rat gastrocnemius PC concentration in the ischemia-reperfusion group when analysed against a control. Conversely, Ozkan et al. (2012) found no significant difference in PC concentrations in rat tibialis anterior between control and ischemia-reperfusion condition subsequent to 3 hrs ischemia and 15 min reperfusion. However, the ischemic-reperfusion condition did display a trend for increased PC concentrations in comparison to control and may be due to a fairly low participant number (n = 6 control, n = 10 ischemia-reperfusion).

The evidence outlined above demonstrates ischemia-reperfusion as a key player in macromolecule damage and that varied durations of ischemia (1.5 – 3 hrs) all provoke disruption in cellular structures. Thus, assessment of macromolecule damage would provide a useful marker in measuring consequent effects of improvements in redox disruptions following the hypoxic intervention.

2.2.4.1 Apoptotic Cell Death

Excessive macromolecule damage via augmented free radical production commences activation of the intrinsic apoptosis pathway, through the initiation of energy-dependant cascades (Elmore, 2007). Oxidative stress mediated DNA

damage stimulates the tumor suppressor protein, p53, to initiate either DNA repair or, stimulate the apoptotic cascade via trans-activation of the large Bcl-2 family (Elmore, 2007; Kroemer et al., 2007). The Bcl-2 protein group consist of both pro- (Bax, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-x) members and are maintained in a hierarchical model (Liu et al., 2010). P53's induction of Bax motivates the pro-apoptotic protein to bind to the mitochondrial outer membrane inducing mPTP, either alone or with other Bcl-2 pro-apoptotic members, thus allowing translocation of very large molecules (Kuwana et al., 2002). The large pore alters the permeability of the mitochondrial membrane, disrupting ATP synthesis and inducing an influx of Ca^{2+}, releasing cytochrome c subsequently promoting the formation of the caspase-activating Apaf-1 apoptosome (Crompton, 1999; Hill et al., 2004). Interestingly, Ca^{2+} influx may not be directly accountable for mitochondrial dysfunction as inhibition of calpain (downstream Bid cleaver) offers myocardial protection from IRI (Chen et al., 2002). The apoptosome triggers caspase-9 to become active, initiating caspase 3 leading to eventual cellular organelle proteolysis, DNA fragmentation and decisive phagocytosis of the apoptotic bodies (Elmore, 2007).

There is an array of evidence to link ischemia-reperfusion with cellular apoptosis. *Ex-vivo* rat hearts participated in 15 min ischemia and 60 min reperfusion, demonstrating large levels of apoptotic cell death in comparison to control and that blockade of oxidative stress upon reperfusion diminished programmed cell death (Maulik et al., 1998). These results have also been collaborated by Galang et al. (2000). Galang and colleagues (2000) showed a dramatic increase in apoptotic *ex-vivo* myocardiocytes after 30 min ischemia and 120 min reperfusion

in comparison to control and that the addition of SOD diminished this negative consequence. However, apoptosis is an energy-dependant process and prolonged exposures to ischemia can lead to vast ATP depletion (Kim et al., 2003; Elmore, 2007). If enough of the cells' mitochondria are damaged, necrotic death will ensue rather than the favourable apoptosis (Yang et al., 2010). Nevertheless, there is no precise feature for either apoptosis or necrosis as both share similar mechanistic pathways, invariably creating difficulty when distinguishing between the two (Jaeschke and Lemasters, 2003).

Apoptosis is cited to play a role in the pathophysiology of skeletal muscle cell loss (Dirks and Leeuwenburgh, 2002) and occurs during ischemia reperfusion following cellular lipid peroxidation (Maulik et al., 1998). Therefore, an intervention which could attenuate apoptosis during tourniquet mediated IRI may enhance recovery times post TKR surgery.

2.2.4.2 Necrotic Cell Death

Necrosis is characterised by cytoplasmic bulging, plasma membrane rupture and organelle damage, resulting in inflammation due to release of cellular components (Festjens et al., 2006). Tumor necrosis factor-α (TNF-α) produced primarily from activated macrophages can induce either cell survival or cell death depending on the cellular environment via ligand-binding with death receptors, TNF-R1 and TNF-R2 (Chen and Goeddel, 2002; Festjens et al., 2006). Although necrosis and apoptosis have very different outcomes, both can be initiated by TNF-α, depending on caspase inhibition (Vanden Berghe et al., 2004). TNF-α cascade signalling induction of Fas-associated death domain mediates apoptosis (Chen et

al., 2002), while receptor-interacting protein triggers necrotic cell death (Hsu et al., 1996).

TNF has been demonstrated to provoke skeletal muscle injury during ischemia-reperfusion, since antibody blockade of TNF resulted in a reduction in skeletal muscle injury (Gaines et al., 1999). This result is in collaboration with Seekamp et al. (1993) who noted a reduction in skeletal and lung injury through TNF obstruction. However, TNF blockade by Sternbergh et al. (1994) did not present a reduction in skeletal muscle endothelial injury, suggesting that TNF may not play a primary role in IRI injury. Interestingly, post TKR surgery, only moderate rises in cytokine concentration (TNF, interlukin-1) have been observed after 2 hrs reperfusion (Clementsen and Reikeras, 2008), although this moderate accumulation of cytokines is in disagreement with Seekamp et al. (1993). The low levels of cytokine observed by Clementsen and Reikeras (2008) in comparison to Seekamp and colleagues (1993) may in part be due to the large disparity between ischemic durations (78-125 min and 4 hrs respectively).

Skeletal muscle necrosis via IRI has been cited to affect muscle function through decreased muscle twitch and contractual force (Kearns et al., 2001), thus an attenuation of this muscle detriment could improve the surgical outcome of TKR patients.

2.2.4.3 Toll-Like Receptors

Necrotic signalling transduction is not just limited to the death receptor pathway, toll like receptors (TLRs) have also been implicated in invoking IRI. TLRs are a family of leucine rich transmembranal proteins involved in regulating innate and

adaptive immune response to pathogenic invaders (Takeda et al., 2003). TLRs are present on immune cells, such as macrophages (Hadley et al., 2007) and non-immune cells including skeletal muscle (Frost et al., 2006). TLRs have been implicated to participate in the pathophysiology of IRI (Arumugam et al., 2009; Khandoga et al., 2009) with TLR-2 (Favre et al., 2007) and TLR-4 (Kaczorowski et al., 2007) implicated as major participators. HSPs and high mobility group box-1 proteins among others, are considered ligands for TLR-2 and TLR-4, which are secreted into the surrounding environment from stimulated leukocytes (Park et al., 2004; Arumugam et al., 2009). The stimulation of TLRs induce numerous intracellular signalling cascades via mitogen activated protein kinase (MAPKs) and IκB kinase, which are very similar to the interlukin-1 pathway resulting in the stimulation of pro-inflammatory transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) (Takeda et al., 2003; Arumugam et al., 2009). The consequential activation of NF-κB and AP1 induces a plethora of functions including a greater expression of cytokines, adhesion molecules and chemokines (Batra et al., 2011).

Research into TLR-$2^{(-/-)}$ knockout mice has been shown to abolish endothelial dysfunction when compared with wild type mice in cardiac tissue after 30 min ischemia and 1 hr reperfusion (Favre et al., 2007). The reduction in endothelial dysfunction was attributed to a reduction in ROS production and leukocyte infiltration. In fact, this explanation was confirmed by Khandoga et al. (2009). The authors utilised TLR-$2^{(-/-)}$ knockout mice with the addition of mice with mutant TLR-4 receptors and demonstrated that after ischemia-reperfusion a reduction in neutrophil endothelial migration was noticed in comparison to mutant

TLR-4 receptor mice, in addition both TLR-$2^{(-/-)}$ knockout and TLR-4 mutant mice displayed attenuated vascular leakage. Indeed, the use of circulatory TLR-2 antibody inhibitors reduced infarct size and diminished functional capacity in murine hearts post ischemia-reperfusion (Arslan et al., 2010). This suggests that TLR-2 plays an important role with trans-endothelial migration of leukocytes in IRI.

Similar results in IRI were also observed in TLR-$4^{(-/-)}$ knockout mice (Oyama et al., 2004) and blockade of TLR-4 via eritoran (Shimamoto et al., 2006). Although both TLR-4 and TLR-2 appear to be in part responsible for the innate inflammatory response during IRI, the latter is illustrated to have a greater role in neutrophil migration (Khandoga et al., 2009).

The culmination of the factors above, in addition to the tissue hypoxia, could negatively affect wound healing following prolonged tourniquet use (Estebe et al., 2011). Therefore, interventions are required to attenuate the duration of wound healing associated with tourniquet use in TKR surgery.

2.3 Ischemic Pre-Conditioning

To avoid the deleterious effects of IRI multiple strategies have been proposed. Numerous studies have administered supplements to mitigate the damage sustained via IRI (Nedrebo et al., 2003; Ozyurt et al., 2006; Avci et al., 2012; Hori et al., 2013). However, pharmacological interventions must be timed perfectly, as well as administered in correct doses with regards to intravenous administration, to ensure the drug has reached the tissue prior to the ischemic insult and in a significant quantity (Wang et al., 2002). In addition, rigorous testing of novel

pharmaceuticals is required to avoid fatal interactions with anaesthesia (Weldon-Bellville, 1972). Furthermore, many of the trials used for supplementation administration are performed on healthy individuals and the effect of pharmaceuticals upon patients who are morbidly obese (often associated with diabetes type II, cardiovascular disease, sleep apnoea) is not well understood (Wang et al., 2002; Samson et al., 2010). Interestingly, morbidly obese is considered an independent cause of knee osteoarthritis (Samson et al., 2010) and morbidly obese individuals contribute to around 28% of total primary TKR patients (Dehn, 2007), thus a non-pharmaceutical intervention would be beneficial.

In contrast to supplementation, a landmark study conducted by Murry et al. (1986) involved a group of dogs receiving 4 cycles of 5 min coronary occlusion and 5 min reperfusion, prior to 40 min of solid coronary ischemia and a 4 day reperfusion period. Surprisingly, the dogs who received the ischemic/reperfusion cycles displayed a reduction in infract size in comparison to a control group only receiving 40 min occlusion. This technique has been aptly termed ischemic preconditioning (IPC) and since this discovery by Murry and colleagues (1986) many researchers have pursued this phenomenon in various tissues other than cardiac muscle, including hepatic (Yoshizumi et al., 1998), skeletal muscle (Saita et al., 2002), brain (Dawson and Dawson, 2000) and renal tissues (Toosy et al., 1999). Thus, to establish the extent of which the non-invasive hypoxic preconditioning can attenuate tourniquet mediated oxidative stress, a comparison to a previously used non-invasive technique is required.

The mechanism by which IPC protects tissue against IRI is not fully understood (Yang et al., 2010), however, it has been established that the protective effects appear in a biphasic pattern (Das and Das, 2008). The early phase is proposed to last between 2-3 hrs (Yang et al., 2010), while a delayed phase appears 24 hrs after the IPC protocol and can last up to 3 days (Hausenloy and Yellon, 2010) (Figure 2.5).

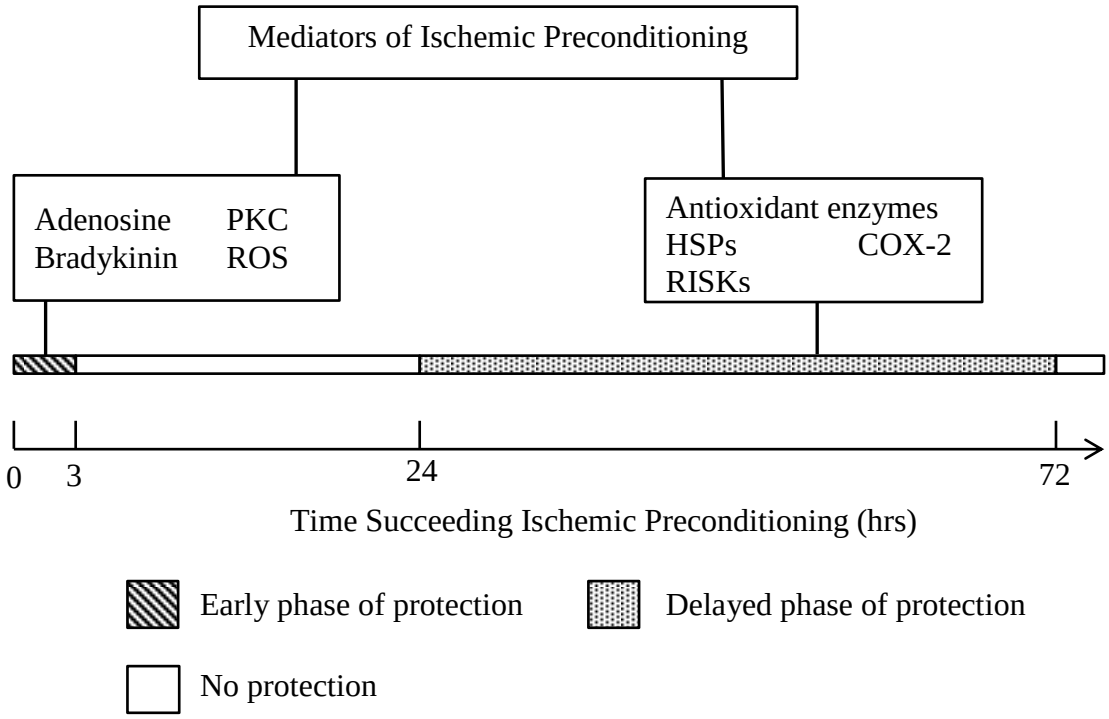

Figure 2.5: Schematic depicting a list of potential mediators and the proposed time frame for the protection offered by ischemic preconditioning. Abbreviations: PKC – protein kinase C; ROS – reactive oxygen species; HSP70 – heat shock protein 70; HSP32 – heat shock protein 32; COX-2 - Cyclo-oxygenase 2; RISKs – reperfusion injury salvage kinases.

2.3.1 Early Phase

Early phase IPC protection has been linked to the activation of G-coupled adenosine receptors via the release of endogenous adenosine during the brief ischemia (Liu et al., 1991). Stimulated adenosine receptors main target during

early IPC is the family of 12 serine/threonine kinases known as protein kinase C (PKC) (Cohen et al., 2000). It has been hypothesised that stimulation of PKC activates 5'-nucleotidase, generating larger concentrations of adenosine via degradation of AMP, which is of abundance during ischemia (Kitakaze et al., 1995). Additionally, PKC is thought to be initiated directly via ROS signalling as intravenous administration of free radical scavengers removes the protection granted from IPC (Baines et al., 1997), while cellular models have demonstrated that the addition of oxidants promotes preconditioning (Vanden Hoek et al., 1998).

Mitchell et al. (1995) demonstrated that PKC activator, 1,2-diacylglycerol (DAG) produced similar cardio-protective effects to IPC in *ex-vivo* rat hearts. Moreover, blockade of PKC abolished the protective effect offered from IPC. The negated effects of IPC were also noted by Ytrehus et al. (1994) following PKC blockade in rabbit hearts. However, the specific PKC isoenzyme that contributes to IPC is still in debate. PKC-$\delta^{(-/-)}$ knockout mice displayed augmented ischemic tissue damage following IPC (Mayr et al., 2004). Conversely, Bright et al. (2004) noted that inhibition of PKC-δ reduced cerebral IRI in rats, indicating the equivocal nature of PKC-δ in inducing IPC. However, it appears that PKC-ε is essential for IPC protection (Ping et al., 2002), and that knockout mice deficient in PKC-ε displayed inhibited protection afforded from IPC (Saurin et al., 2002). Consequently, PKC-δ may not be essential in IPC unlike the isoform PKC-ε.

IPC is proposed to act on the mPTP, offering protection through maintaining inhibition of pore opening (Das and Das, 2008). As previously mentioned, (in

section 2.2.4.1) opening of the mPTP has catastrophic effects upon the mitochondria, with eventual apoptosis ensuing. Mitochondrial potassium ATP (mK_{ATP}) channel activation has been linked to inhibiting mPTP opening, although the mechanisms involved are unclear (Hausenloy et al., 2002). The activation of PKC-ε via adenosine allows the stimulated serine/threonine kinase to open the mK_{ATP} channel (Hu et al., 1999). Influx of K^+ into the mitochondria maintains Ca^{2+} concentrations via reducing the electrochemical gradient for Ca^{2+} movement into the mitochondria (Holmuhamedov et al., 1999) thus restraining Ca^{2+} mediated mPTP opening and subsequent apoptosis. It has also been speculated that PKC-ε may regulate the apoptosis through coimmunoprecipitation to components of the mPTP (voltage dependant anion-selective channel, adenine nucleotide translocase) inhibiting mPTP opening (Yonekawa and Akita, 2008).

Therefore, any immediate reduction in oxidative stress markers from IPC to a subsequent bout TKR specific tourniquet application would involve the mechanisms outlined above.

2.3.2 Delayed Phase

After the initial 2 hr window of protection offered by IPC, the delayed phase takes effect 24 hrs post preconditioning and has been attributed to altered gene expression and *de novo* synthesis of proteins (Carden and Granger, 2000). Mediators of delayed protection are generally protein kinases which are activated during the IPC stimulus resulting in activation of transcription factors (Hausenloy and Yellon, 2010).

The established mediator of early protection, PKC, has also been implicated to produce delayed protection (Qiu et al., 1998). Qiu et al. (1998) demonstrated inhibition of PKC after the ischemic stimulus did not abolish the protection offered by IPC suggesting crucial activation of PKC occurs during the ischemic bout. Qiu and colleagues (1998) also established that general activation of PKC without ischemia mimicked the effects of delayed IPC. MAPKs have also been recognised as delayed protection mediators via upstream stimulation from PKC (Xuan et al., 2005). Indeed, research by Fryer et al. (2001) noted the integral role of extracellular signal regulated kinase (ERK) and p38 MAPK in delayed cardioprotection. The stimulation of mediators initiates activation of a variety of cell stress signalling transcription factors associated with delayed IPC such as hypoxia induced factor -1α (Xi et al., 2004), NF-κB (Xuan et al., 1999) and AP-1 (Li et al., 2000). Although, it has been suggested that transcription factors can be recruited differentially and still induce the requisite delayed protection (Hausenloy and Yellon, 2010). Cell stress transcription factors can be induced via a differing stress (e.g. hyperthermia) but still yield protection from IRI, demonstrating a cross-tolerance effect (Horowitz et al., 2004). Therefore, hypoxic preconditioning could yield delayed protection from ischemia via stressor cross-tolerance.

2.3.2.1 Antioxidant enzymes

Transcriptional mediators of delayed IPC are proposed to up-regulate antioxidant enzymes (Hausenloy and Yellon, 2010), including, peroxisomal catalase, glutathione peroxidase and MnSOD (Das et al., 1993). MnSOD is a key mitochondrial protein which is synthesised in the cytoplasm and imported

posttranslationally into the mitochondria, where it functions to catalyse the dismutation of $O_2^{\bullet-}$ into H_2O_2 and molecular oxygen (Jin et al., 2005). Dramatic increases in MnSOD have been noted 24 hrs following a bout of IPC in canine hearts (Hoshida et al., 1993). Indeed, Zhou et al. (1996) also demonstrated greater MnSOD induction and activity 24 hrs following IPC in rat myocytes in comparison to control. However, not all research has verified increases in MnSOD activity succeeding IPC. Tang et al. (1997) showed that delayed IPC did not increase MnSOD activity in porcine hearts. Nevertheless, the discrepancy in results observed by the various research groups may be attributable to specie or IPC protocol differences. Regardless of these variances, it is likely that an increase in MnSOD following IPC would partially augment protection from future ischemic insults.

2.3.2.2 Heat shock protein 72

Marber and colleagues (1993) were the first to illustrate an increase in HSP72 following IPC (4 x 5 min ischemia interrupted with 10 min reperfusion) 24 hrs earlier in cardiac tissue, with the rise in HSP72 associated with the mechanisms involved in delayed preconditioning. HSPs are a family of highly conserved cytoprotective proteins ubiquitously found in cells, which function as molecular chaperones facilitating folding and transportation of newly synthesised and denatured proteins (Morton et al., 2009). In addition, HSPs play a versatile role in cellular survival via interaction with molecules associated with the apoptotic cascade (Kalmar and Greensmith, 2009). HSPs are defined by their estimated molecule weight and tend to be grouped into sub-group families (i.e. HSP70 (70 kDa)), and are localised throughout various regions of the cell (Morton et al.,

2009). HSP70 in particular, is highly inducible during stressful situations, rescuing damaged proteins and maintaining cellular protein synthetic capacity (Noble et al., 2008).

The coordinated organisation of activated HSP gene expression (Hsp) in response to a variety of stressors, including ischemia (Chang et al., 2001), hyperthermia (Oishi et al., 2003) and hypoxia (Taylor et al., 2010) is called the heat shock response. Under normal physiological conditions, HSP70 is bound to its transcription factor, heat shock factor 1 (HSF1), in an inactive monomeric state (Abravaya et al., 1992). However, an external stressor induces intracellular protein unfolding and denaturation, stimulating the dissociation of the HSP70 and HSF1, through HSP70 preferential binding to the damaged protein (Noble et al., 2008). Unbound, HSF1 undergoes trimerisation with other free HSF1, subsequently binding to the heat shock element in the promoters of heat shock genes, allowing transcription of additional HSPs (Sarge et al., 1993) (Figure 2.6).

The integral role of HSP72 during cellular stress and its rapid induction via a variety of stressful stimuli make it an excellent marker of redox disturbance (Taylor et al., 2012). Indeed, oxidative stress has been cited to up-regulate HSP72 via signal activation of HSF1 and by directly oxidising cellular proteins (Morton et al., 2009). Therefore, a rise in Hsp72 would indicate greater cellular stress following the TKR specific tourniquet application.

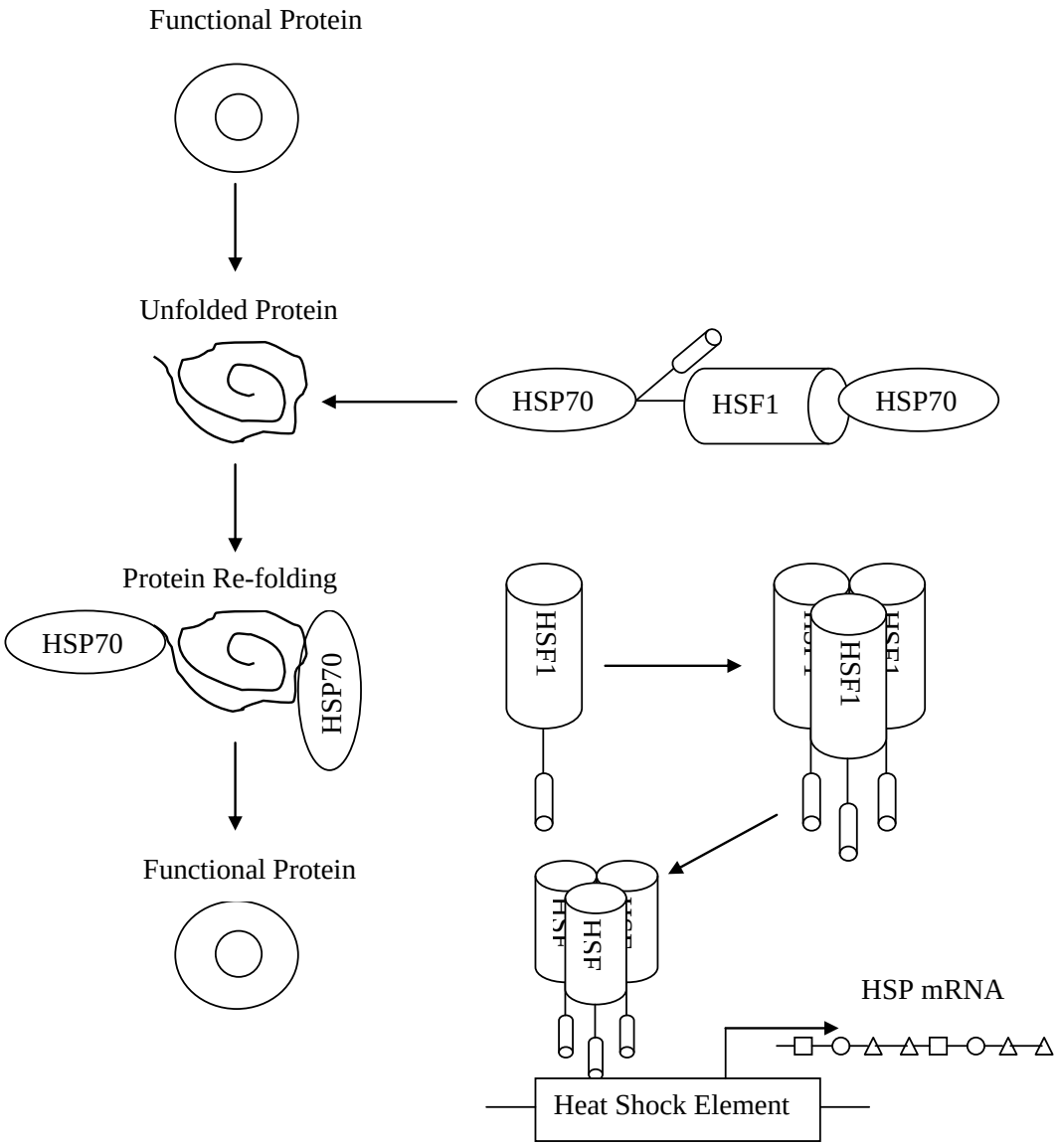

Figure 2.6: Stress induced activation of the heat shock response mechanism. Unfolded proteins induce the dissociation of HSP70 from its bound monomeric state with HSF1, allowing HSP70 to aid in the refolding of damage proteins. Unbound HSF1 trimerises with other free HSF1, subsequently binding the heat shock element, initiating transcription. Abbreviations: HSP70 – heat shock protein 70; HSF1 – heat shock factor 1. Adapted from Nobel, Milne & Melling (2008).

Since the original observation between delayed IPC and HSPs by Marber et al. (1993) subsequent research groups have explored this connection. Okubo et al. (2001) injected recombinant adenovirus encoding for HSP70 into *in-vivo* rabbit hearts 4 days prior to 30 mins cardiac ischemia and 3 hrs reperfusion. The authors

noted a reduction in infarct size in the HSP70 vector comparison to a saline injection alone. This evidence for the role of HSP70 in delayed IPC was also observed by hyperthermic preconditioning (Lepore et al., 2000). Lepore and colleagues (2000) performed hind limb heating at 42°C for 20 min prior to 2 hrs ischemia and 24 hrs reperfusion. The passive heating bestowed a rise in HSP72 and subsequent protection from the ischemic insult. Interestingly, Tanaka et al. (1998) observed increases in HSP70 after just 3 hrs following IPC in rabbit hearts, with concentrations remaining detectable by immunohistochemical techniques for up to 72 hrs. In contrast to this, Lepore and Morrison (2000) did not observe an increase in HSP70 gene expression from 2 x 10 min bouts of ischemia with 15 min reperfusion interruptions in rat hind limb skeletal muscle. Additionally, the authors noted that there was not a significant increase in viable fibres following 2 hrs of ischemia in comparison to a control group. However, the poor sample number (n = 3) and altered preconditioning protocol to previous successful work (Marber et al., 1993) may have led to the non-significant result ($p = 0.16$).

Interestingly, repeated bouts of endurance exercise can provide a marked rise in HSP72 within cardiac tissue (Powers et al., 1998; Demirel et al., 2003) inducing protection from IRI (Steel et al., 2004). However, HSP72 is not necessarily required to attenuate IRI. Research from Taylor et al. (1999) and Hamilton et al. (2001) both demonstrated that exercise bestowed protection from IRI without an increase in cardiac HSP72. It is possible that the disparity between cardiac and skeletal muscle research may be attributable to varying degrees of HSP72 dependency in differing tissues. Indeed, Hamilton et al. (2001) postulated that protection in cardiac tissue mediated via exercise may be partly due to greater

antioxidant capacity. It could therefore be speculated that cardiac tissue relies more heavily on antioxidant enzymes in comparison to skeletal muscle which may depend more on HSPs, although further research is necessary.

In summary, HSP72 appears to play a key role in skeletal muscle delayed IPC (Marber et al., 1993; Okubo et al., 2001); therefore a deferred increase in Hsp72 from IPC may indicate the onset of delayed preconditioning.

2.3.2.3 Heat shock protein 32

HSP32 (or Heme oxygenase-1) is another member of the cytoprotective family associated with delayed IPC (Hausenloy and Yellon, 2010). Free heme is bound in hemoproteins during homeostasis, however, during oxidative stress, free heme molecules are released from heme pockets within the hemeproteins and in doing so produce ROS via Fenton chemistry (Gozzelino et al., 2010). HSP32 is an evolutionary conserved enzyme that catabolises the ROS producing free heme into biliverdin, carbon monoxide (CO) and iron, (Tenhunen et al., 1968). Bilverdin is then available for enzymatic conversation via bilverdin reductase (BVR) into the cytoprotective anti-oxidant bilirubin (Clark et al., 2000).

HSP32 expression is regulated by numerous stress-associated effectors such as heme, hyperthermia, and hypoxia (Alam and Cook, 2007). Transcriptional activation of HSP32 is controlled by a variety of transcription factors including, nuclear factor E2-related factor 2 (NrF2), NF-κB, AP1 and HSF-1 (Kim et al., 2011). Stressful circumstances have the ability to stimulate multiple transcription factor groups simultaneously, with differing group's regulating slightly different aspects in HSP32 stress response (Alam and Cook, 2007). The MAPK cascade

appears to be the foremost mediator of HSP32 gene regulation (Figure 2.7), involving the three major sub-families, ERK, c-Jun N-terminal kinases (JNKs) and p38 kinases (Alam et al., 2004). Indeed, ERK, JNKs and p38 MAPK were all attributed to the upregulation of HSP32 following IRI (Zhang et al., 2002).

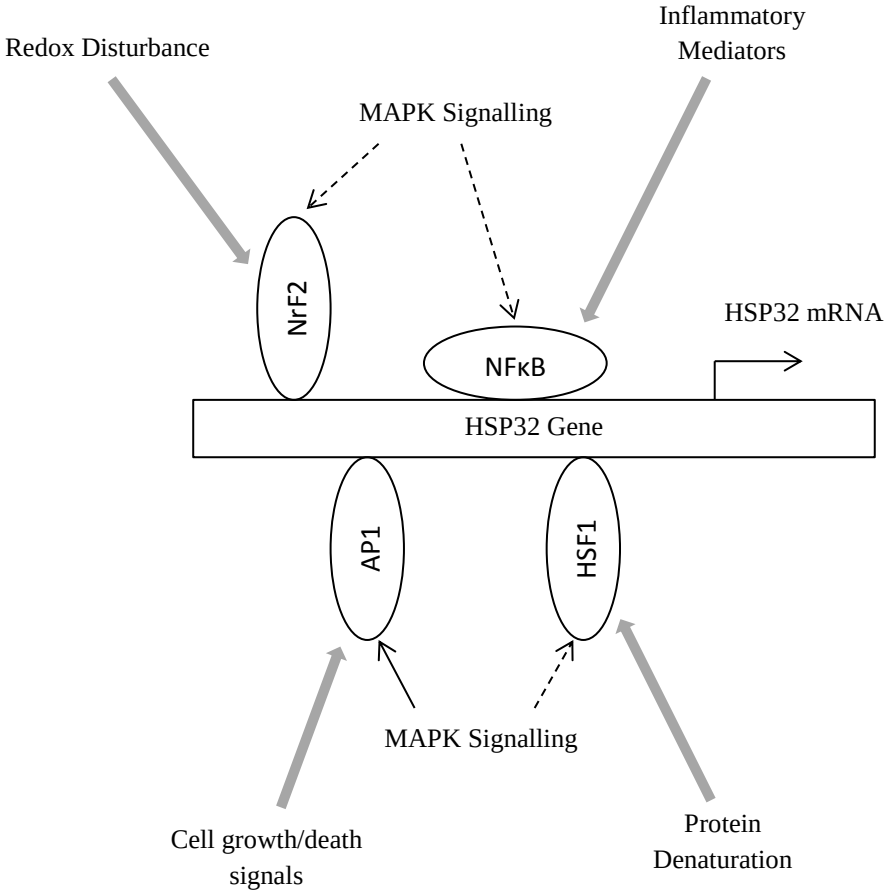

Figure 2.7: Regulation of the HSP32 gene via the major stress-response transcription factors. Stimulation of transcription factors occurs through MAPK signalling (direct phosphorylation (solid line); signalling cascade (dotted line)) or directly by stressful stimuli (grey line). Abbreviations: NrF2 – Nuclear factor E2-related factor 2; NF-κB – Nuclear factor-κB; AP1 – Activator protein 1; HSF1 – Heat Shock factor 1; MAPK – Mitogen activated protein kinases; HSP32 – Heat shock protein 32. Adapted from Alam & Cook (2007).

Similar to HSP72, the inducible form of HSP32 has also been demonstrated to be a marker of oxidant mediated cell damage (Rothfuss et al., 2001). Thus, an increase in Hsp32 following tourniquet application, would indicate a rise in oxidative stress.

Myocardial cells undergoing IPC displayed an increase in HSP32 24 hours post ischemic stimulus, in addition it to an increase in cell viability in comparison to a simulated ischemic group (Jancso et al., 2007). Further evidence for HSP32's induction through IPC was noted by Zeynalov et al. (2009), who performed IPC on wild type mice and found a significant increase in protein expression 24 hours post stimulus in comparison to a sham control. Furthermore, the anti-oxidant by-product of HSP32 activity, bilirubin, was noted to be significantly increased in IPC rat skeletal muscle in contrast to control (Badhwar et al., 2004).

2.4 Hypoxic Preconditioning

HPC has been utilised previously to bestow cellular tolerance as a stratagem to avoid deleterious effects from disturbances to the redox balance (Taylor et al., 2012). Moreover, hypoxic exposure has been described to have similar protective effects as IPC in providing tolerance to a subsequent sustained bout of ischemia (Beguin et al., 2005). In line with IPC, HPC also offers biphasic protection at similar intervals (i.e. within 2 hrs and between 24-72 hrs of stimulus removal) (Zhao et al., 2013). Furthermore, both IPC and HPC appear to share similar redox pathways in response to hypoxia mediated oxidative stress (Zuo et al., 2013). PKC-ε is attributed to an integral role in inducing early IPC, but the kinase has additionally been demonstrated to be up-regulated following HPC and that general

blockade of all PKC isoforms abolish the protection conferred by the hypoxic exposure (Berger et al., 2010). In addition, it has been shown that the increase in ROS via HPC initiates ischemic tolerance (Vanden Hoek et al., 1998), perhaps via ROS mediated stimulation of PKC. Indeed, PKC-ε has also been shown to up-regulate HSP32 (Ryter et al., 2006), further elucidating the vital role of PKC-ε in HPC. Thus, the HPC undertaken in the present thesis may offer protection to tourniquet mediated oxidative stress immediately following the hypoxic exposure.

The delayed phase of HPC operates in an identical manner to IPC by initiating *de novo* synthesis of proteins (Zhao et al., 2013). HPC has been speculated to activate the transcription factor, hypoxia induced factor -1α, which stimulates up-regulation of target genes including, vascular epidermal growth factor, inducible nitric oxide synthase and erythropioetin (Ran et al., 2005). These genes would promote greater blood flow and oxygen delivery for subsequent ischemic insults (Ran et al., 2005). Moreover, Taylor et al. (2010) noted an increase in monocyte HSP72 gene expression following a 75 min period of normobaric hypoxia (14.5% O_2) *in vivo*. Indeed, HPC mediated up-regulation of HSP72 has been demonstrated to diminish the deleterious effects of IRI in the rat kidney (Yeh et al., 2010). Furthermore, HPC has been elicited to increase HSP32 following a prolonged hypoxic exposure, attenuating hepatic IRI in rats (Lai et al., 2004).

The mechanisms outlined above describe the process in which HPC could potentially protect skeletal muscle against IRI following TKR specific tourniquet application. Therefore, a reduction in redox mediated tissue damage could lead to less wound complications and greater surgical outcomes (Soneja et al., 2005; Estebe et al., 2011).

Mice kept in normobaric hypoxic (10% O_2) chambers for 4 hrs followed by 24 hrs in normoxia prior to 20 min global cardiac ischemia and 30 min reperfusion, displayed a reduction in infarct size in comparison to control mice in isolated hearts (Xi et al., 2002). Furthermore, HPC prior to 6 hrs of hypoxia (0.5% O_2) and 12 hrs re-oxygenation provided a significant reduction in apoptosis of mesenchymal cells *in-vitro* (Wang et al., 2008). Further *in-vitro* work by Wu et al. (2013) illustrated that 20 min of HPC simulated by incubation in anoxia (95 % N, 5% CO_2) 24 hrs prior to 3 hrs of anoxia induced a reduction in ECs apoptosis. Animal *in-vivo* research by Berger et al. (2010) performed 4 hrs of systemic normobaric hypoxia at different concentrations (8%, 12% and 16% O_2) in rats. The author noted a significant reduction in infarct size in all HPC condition in comparison to control.

In summary, IPC has been shown on numerous occasions to diminish the deleterious effects of IRI in a multitude of tissues, with similar results observed in HPC. However, there is a lack of research regarding the effects of HPC in human *in-vivo* skeletal muscle and circulation following TKR specific tourniquet mediated limb ischemia.

Chapter 3: Methodology

3.1 Participants

Eighteen recreational healthy male participants were recruited for the study (see Table 3.1 for demographic data). Prior to experimental contribution, participants were all informed of the procedure and the risks involved in participating, subsequently providing written informed consent, according to the principles set out in the Declaration of Helsinki (Appendices B - E). Each participant was medically screened and ethical approval was obtained via the University of Bedfordshire's, Sport and Exercise Science Department Human Ethics Committee. It was mandatory that participants were free from any musculoskeletal injury or acute or chronic sickness, in addition to abstinence from medication (vitamin supplementation), ergogenic aids (creatine, ß-alanine,) and extreme environments (hyperthermia, hypoxia) that may affect any variables intended to be measured in this study. All participants were non-smokers and 72 hours prior to testing, participants were required to refrain from alcohol, caffeine and exercise (the full list is available in Appendix A). Participants were randomly allocated into either control (SHAM), hypoxic preconditioning (HYP) or ischemic preconditioning (TOR) conditions.

3.2 Anthropometric Data

Body mass (kg), and height (m) were assessed with the use of a Digital Tanita scales (BWB0800, Allied Weighing, UK) and a wall-mounted Stadiometer (Holtain Ltd, UK) respectively. Participants were asked to refrain from fluid/food ingestion 4 hours, in addition to an evacuated bladder immediately prior to estimation of percentage muscle and fat mass (%) through utilisation of air

pletysmography (BodPod 2000A, Cranlea, UK). Furthermore, individual's blood pressure was noted in triplicate and an average was obtained (M5-I, Omron, Cranlea, UK). Participants were seated for 5 min and requested to close their eyes and relax prior to measurement acquisition based on the manufacturer's instructions. Finally, thigh circumference was obtained in triplicate using a tape measure (Body Care, HaB Direct, UK) at 40% of the distance from the knee joint centre to the greater trochanter on the right leg (Table 3.1).

Table 3.1: Participant demographic data

Measure	SHAM			HYP			TOR		
	Mean	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**
Age (years)	22.2	2.9	18 - 26	20.8	2.4	19 - 25	18.5*	0.6	18 - 19
Height (m)	1.83	0.06	1.75 - 1.92	1.77	0.10	1.67 - 1.93	1.79	0.04	1.73 - 1.83
Mass (kg)	80.4	12.4	62.8 - 93.4	73.5	8.7	61.9 - 86.6	76.7	7.8	64.8 - 87.4
Lean mass (%)	85.8	3.5	79.3 - 88.5	84.9	5.3	78.9 - 91.4	86.5	3.0	81.5 - 90.1
Fat mass (%)	14.2	3.5	11.5-20.7	15.3	5.5	8.6 - 22.1	13.5	3.0	9.9 - 18.5
Thigh Circumference (cm)	44.7	2.6	40 - 47	42.8	2.1	40 - 46	43.3	2.4	39 - 46
Systolic Blood Pressure (mmHg)	124.0	3.0	120 - 129	125.8	1.9	123 - 129	125.5	2.1	123 - 129
Diastolic Blood Pressure (mmHg)	76.2	7.0	65 - 83	75.0	6.0	65 - 81	79.7	8.0	70 - 92

* Significant difference v.s. SHAM ($p < 0.05$)

3.3 Hydration Status Assessment

Dehydration has the potential to induce oxidative stress (Paik et al., 2009), which in turn initiates regulation of HSPs (Ahn and Thiele, 2003). Therefore, the author felt it necessary to assess hydration status prior to commencing the trials in order to minimise erroneous results. Urine refractormetry has been previously shown to provide a reliable measure for urine specific gravity to assess hydration status (Stuempfle and Drury, 2003).

Upon arrival to the laboratory, participants were requested to provide a urine sample for analysis via a urine refractometer (Pocket Pal-Osmo, Atago Vitech Scientific, HAB Direct, UK). Samples were assessed in triplicate and euhydratrion was accepted at 200-600 mOsmols·kgH_2O^{-1} utilised previously by (Hillman et al., 2011)

3.4 Blood Collection

Blood samples were obtained using Vasoplus Needles (22G x 1 ½", Grenier Bio-One, UK) from the antecubital region via standard venepuncture techniques (Figure 3.1). Samples were drawn directly into three separate vacuette containers (Vacuette®, Grenier Bio-One, UK) treated with K3EDTA (HSP gene expression (Hsp)), sodium citrate (TGSH, GSSG) or lithium heparin (PC).

Figure 3.1: Image of the venepuncture technique used during the study.

The author originally proposed to acquire blood samples from the ischemic lower leg, in addition to blood from the antecubital region, as previous research (Karg et al., 1997; Garcia-de-la-Asuncion et al., 2012) has shown differing metabolite concentrations in systemic and localised blood following limb ischemia. However, following pilot-work, it was established that obtaining blood from the lower leg was not possible and only systemic blood was collected thereafter.

3.4.1 K_3EDTA Treated Blood

Blood treated with the anti-coagulant EDTA has been demonstrated to increase the yield of Hsp concentrations in comparison to alternative method (Whitham and Fortes, 2006). Leukocytes were isolated utilising an adaptation of a technique previously validated (Sandstrom et al., 2009; Taylor et al., 2010; Hillman et al., 2011).

Briefly, 1 mL of K_3EDTA blood was added to 1:10 eythrocyte lysis solution (Miltenyi Biotec, UK) and allowed to incubate at room temperature for 15 min, prior to isolation via centrifugation at 400 G for 5 min at 4°C. Supernatant was removed and the remaining pellet was washed with 2 mL of phosphate buffered saline (PBS) solution (Fisher Scientific, UK) then centrifuged at 400 G for 5 min at 4°C. Supernatant was discarded and a repeat wash was performed. The pellet was suspended in 1 mL of PBS and separated equally into two 1.5 mL RNase free eppendorfs then centrifuged at 17 000 G for 5 min at 4°C. The remaining supernatant was aspirated prior to the pellet being completely re-suspended in 200 µL of TRIzol reagent (Sigma Aldrich, Dorset, UK) and stored at -80°C for subsequent RNA extraction (section 3.10).

3.4.2 Sodium Citrate Treated Blood

Two mL of sodium citrate blood was immediately added to 8 mL of freshly prepared 5% metaphosphoric (Sigma Aldrich, Dorset, UK) and left to incubate on ice for 15 min before being centrifuged at 12 000 G for 15 min at 4°C. The clarified supernatant was collected and separated into 1.5 mL eppendorfs prior to

storage at -80°C until future analysis for TGH and GSSG with commercially available kits (section 3.9).

3.4.3 Lithium Heparin Treated Blood

The collected blood was immediately centrifuged at 900 G for 10 min at 4°C before the plasma was separated into 1.5 mL eppendorfs and stored at -80°C until future analysis via commercially available kits (section 3.8).

3.5 Muscle Biopsies

Muscle Biopsies were obtained from the lateral head of the gastrocnemius of the ischemic leg 2 cm apart under local anaesthetic (2% lidocaine hydrochloride), specifically avoiding the fascia of the muscle as outlined by Trappe et al. (2013), using a disposable biopsy needle (Figure 4.2) (12 x 16, Disposable Monopty Core Biopsy Instrument, Bard Biopsy Systems, USA) and placed into 2 mL RNase free tubes (detailed method in Appendix F). Multiple sample sites were elected as previous research has shown multiple passes from a single incision can alter tissue gene expression (Friedmann-Bette et al., 2012). Samples collected (20-30 mg) were immediately frozen in liquid nitrogen (-196°C) and stored at -80°C for subsequent RNA extraction (see section 3.10). Serial muscle biopsies have been previously demonstrated not to provoke stress proteins in the residual tissue (Khassaf et al., 2001).

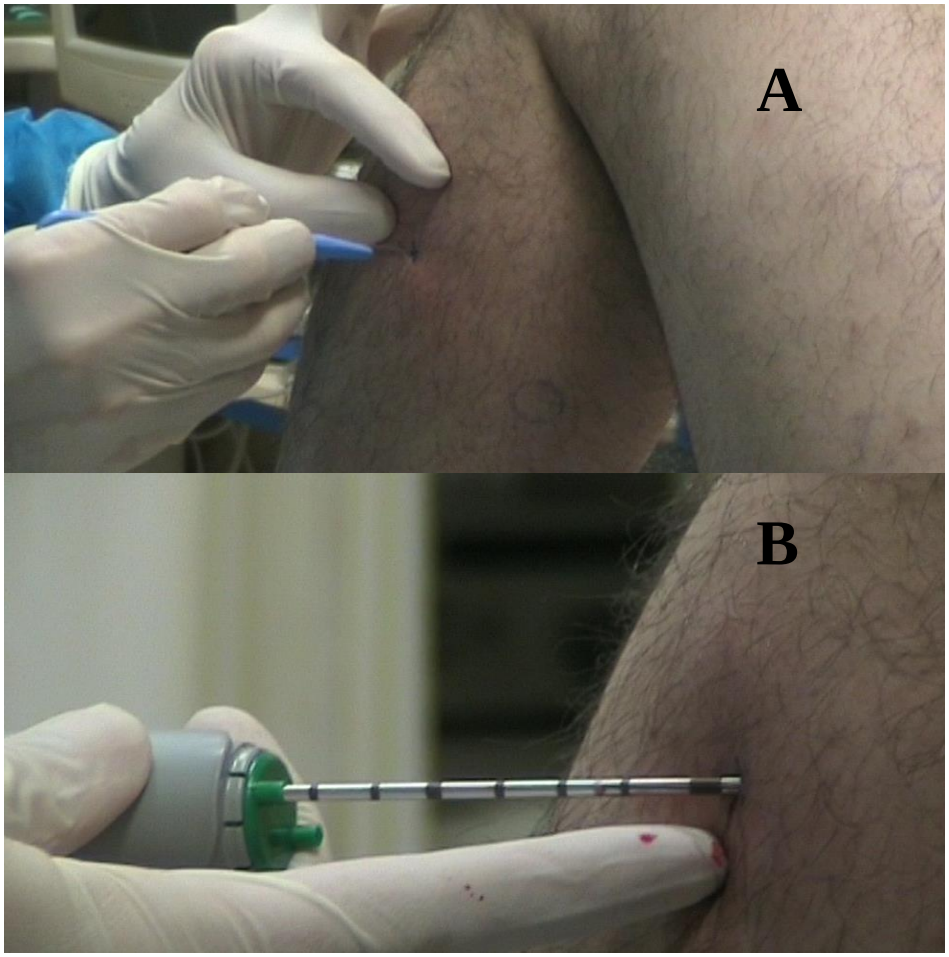

Figure 3.2: Images of the muscle biopsy procedure. (A) The gastrocnemius was cleaned with cyclohexane, injected with lidocaine subsequently a small incision was made. (B) 20-30 mg of tissue was obtained using Bard Biopsy needle.

3.6 Experimental Design

Participants arrived at the University of Bedfordshire's Sport and Exercise Laboratories for two separate visits at 11:30. The laboratories were maintained at a constant temperature (mean ± SD; 22 ± 1°C) throughout the entire experiment. The first visit was used to provide anthropometric data as outlined in section 3.2 and occurred between 7-14 days prior to the second visit. Participants were

requested to consume a standardised meal in the evening prior to their second visit.

Upon arrival to the laboratories for the second time, participants were requested to provide a urine sample and abstinence information as depicted by section 3.3 and 3.1 respectively, in addition to providing a food diary for the past 3 days ingestion. Participants were positioned in an inclined supine position throughout the trial and asked to move as little as possible. Participants were permitted a standardised breakfast and lunch 3 hrs prior to commencing the trial and 2 hrs into experiment respectively. Compliance was monitored via a questionnaire. The author would have preferred participants to be fasted for 6 hrs and be nil-by-mouth for the duration of the trial to simulate hospital protocol. However, pilot-work demonstrated that due to the invasive nature of the study, individual's suffered from bouts of syncope during the muscle biopsy procedure, thus making this unethical.

All participants were initially rested for 1 hour prior to undertaking their allocated 40 min preconditioning intervention. Individuals in the SHAM condition received an extended rest period. HYP inhaled 14.3% O_2 (2, 980 m above sea level) in normobaric pressure via an adjustable hypoxicator (Everest Summit II, The Altitude Centre, UK), which has been shown to be an adequate stimulus to induce a cellular stress response *in-vivo* (Taylor et al., 2010). The hypoxicator produces the necessary hypoxic load via O_2 filtration. During the hypoxic exposure, participants heart rate (HR) and oxyhaemoglobin saturation were measured every 5 min via finger pulse oximetry (Onyx® II 9550, Nonin Medical, USA). TOR

received 4 cycles of 5 min ischemia and 5 min reperfusion at 100 mmHg above the participant's systolic pressure on their right leg. The chosen pressure was based upon previous research by Estebe et al. (2000), with the duration of ischemic cycles adapted from work by Koca et al. (2011). All ischemic bouts throughout were produced via a straight 10 cm wide tourniquet cuff (AET, Anetic Aid, Leeds, UK) positioned superiorly to cotton wool padding (Estebe et al., 2011), with pressure maintained by means of an electronic tourniquet unit (AET, Anetic Aid, Leeds, UK).

Upon cessation of the preconditioning intervention, participants rested again for 1 hour prior to a 5 min 45° limb elevation, immediately followed by a 30 min tourniquet application (100 mmHg above resting systolic pressure) on the right leg and a 2 hour period of reperfusion. The tourniquet pressure of 100 mmHg above systolic was elected as it is sufficient to provide a bloodless field while reducing the likelihood of associated negative side-effects (Worland et al., 1997).

Blood samples were obtained at Basal, immediately post intervention (PoI), immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT) and 120 min post tourniquet removal (120PoT) utilising the procedure and controls outlined in section 3.4, with the addition of muscle samples collected at PrT, 15PoT and 120PoT in accordance with section 3.5 (Figure 4.3).

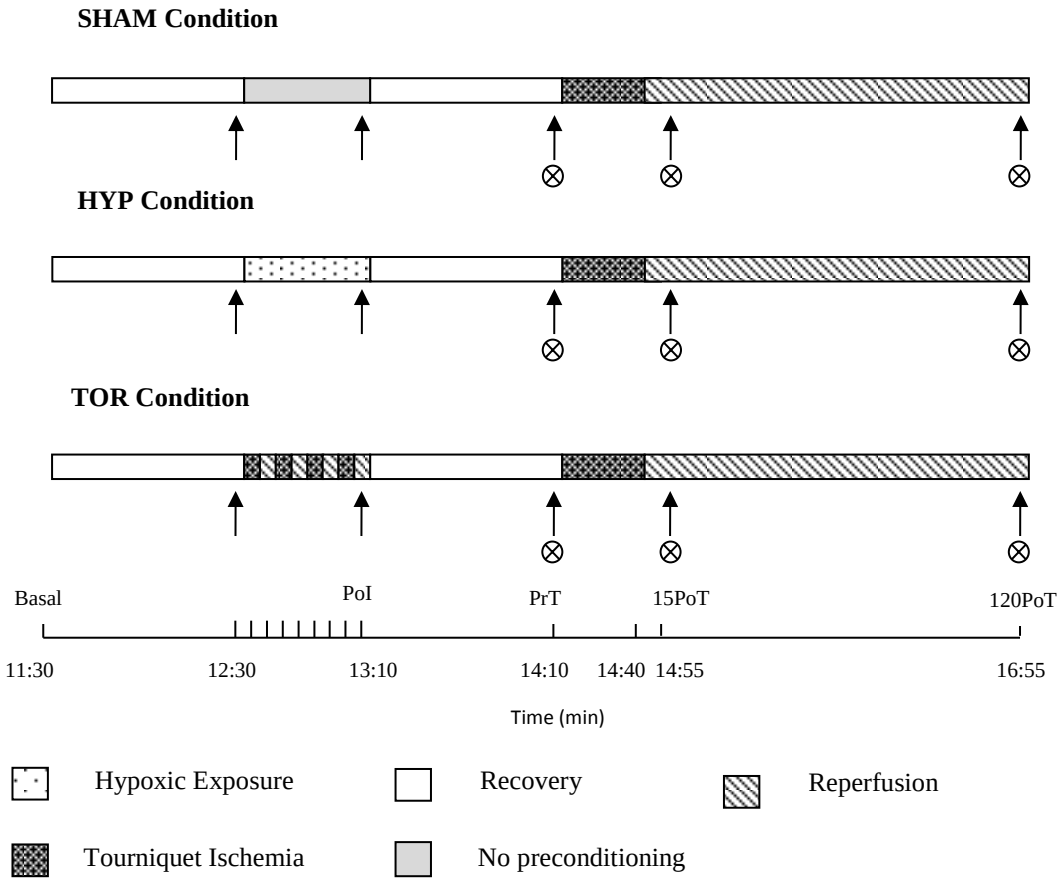

Figure 3.3: Experimental design for all conditions. Blood samples (↑) were obtained at Basal, PoI, PrT, 15PoT and 120PoT with additional gastrocnemius tissue (⊗) collected at PrT, 15PoT and 120PoT.

3.7 Muscle Sample Preparation

Muscle samples were ground under liquid nitrogen prior to homogenisation (T10 Basic, IKA, Thermo Fisher Scientific, Loughborough, UK) on ice in 1 mL TRIzol reagent followed by a 10 min incubation period on ice. RNA was extracted utilising the method described in section 3.10.

3.8 Protein Carbonyl Quantification

PC is a widely accepted measure of protein oxidation (Powers et al., 2010b) and has been cited as more stable marker of oxidised structural modifications in

comparison to the more commonly used transient measures of MDA (Pantke et al., 1999), therefore, this particular marker was chosen and analysed using commercially available kits (Protein Carbonyl Colorimetric Assay Kit, 10005020, Caymen Chemical Company Company, Michigan, USA).

Two hundred µL of pre-treated lithium heparinised plasma (section 3.4.3) was added to 800 µL of 2,4-dinitrophenylhydrazine acting as the sample tube while 200 µL of plasma was added to 800 µL of 2.5 M hydrochloric acid to serve as the control tube. All tubes were required to incubate in the dark for 1 hour at room temperature with a brief vortex every 15 min. 1 mL of 20% trichloroacetic acid (TCA) was added to each tube, briefly vortexed and incubated on ice for 5 min prior to centrifugation at 10 000 G for 10 min at 4°C. This was followed by a 10% TCA wash, incubation on ice for 5 min and centrifuged at 10 000 G for 10 min at 4°C. Supernatant was discarded and the pellet suspended in a 1:1 ethanol/ethyl acetate wash before undergoing a thorough vortex and centrifugation at 10 000 G for 10 min at 4°C. This was repeated twice more before the pellet being re-suspended in 500 µL of guanidine hydrochloride and centrifuged at 10 000G for 10 min at 4°C. An aliquot of 220 µL of both sample and control was added to a 96-well plate and the absorbance was measured at 360 nm using a microplate reader (Sunrise™, Tecan, Reading, UK). All samples and standards were analysed in duplicate.

PC concentration was analysed using the subsequent equation:

$$PC\ (nmol \cdot mL^{-1}) = \frac{CA}{0.011\ \mu M^{-1}} \times \frac{500\ \mu M}{200\ \mu M}$$

Corrected absorbance (CA) was produced through the subtraction of the average control absorbance from the average sample absorbance with 500 µL/200 µL providing the original sample concentration and 0.011 μM^{-1} as the actual extinction coefficient for 2,4-dinitrophenylhydrazine at 370 nm. The intra and inter-assay coefficient of variance are 4.7% and 8.5% respectively.

To assess the protein content of the sample, a 1:10 dilution of sample control to gunadine hydrochloride was prepared, the absorbance was determined at 280 nm using a Nanodrop 2000c (Thermo Fsiher Scientific, Loughborough, UK) and calculated from a bovine serum albumin standard (0.25-2.0 $mg \cdot mL^{-1}$) curve using the following equation:

$$Protein\ Concentrations\ (mg \cdot mL^{-1}) = \left(\frac{A_{280}\ -\ Y_{Intercept}}{slope}\right) \times 2.5\ \times 10$$

The final assessment of carbonyl content is produced via the subsequent equation:

$$Carbonyl\ Content\ (nmol \cdot mL^{-1}) = \frac{Carbonyl\ (nmol \cdot\ mL^{-1})}{Protein\ (mg \cdot mL^{-1})}$$

3.9 Glutathione Analyses

Glutathione has been reported to provide a useful marker for disturbances to the redox balance (Powers et al., 2010b). The most commonly used technique is high performance liquid chromatography; however, this involves large quantities of time pre- and post-assay procedure (Asensi et al., 1994) and can involve varying degrees of GSH recovery (Ostman et al., 2004). Spectrophotometric techniques are often utilised via verification of glutathione in the 'recycling method' of Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid (DTNB)) and GSH

measuring absorbance at 412 nm; providing convenience, sensitivity and accuracy in various sample types (blood, urine, muscle, liver) (Rahman et al., 2006), thus for these reasons, this method was chosen for this research project.

To determine the concentration of TGH previously treated blood (50 µL; section 3.4.3) was diluted to 1:40 with assay buffer solution and transferred to a 96-well plate in accordance with the manufacturer's instructions (Glutathione (Total) Detection Kit, ADI-900-160, Enzo Life Sciences, Exeter, UK). A standard curve was created through serially diluting 50 µL GSSG standard and 50 µL of assay buffer solution (100-12.5 pmol). A 150 µL mixture of DTNB and 10 µL glutathione reductase was added to all wells to produce 5-thio-2-nitrobenzoic acid (TNB) which measured absorbance at 405 nm in a microplate reader (Sunrise™, Tecan, Reading, UK) every minute for 10 min. For determination of GSSG, the method outline above was replicated with the addition of samples first being treated with 1 µL of 2M 4-Vinylpyridine (Sigma Aldrich, Dorset, UK) to block any free thiols from cycling the reaction. 4 µL of 2M 4-Vinylpyridine was added to 200 µL of GSSG standard to produce a standard curve. Samples and standards were incubated for 1 hr and analyses were identical to the protocol for TGH. Reduced glutathione was calculated via subtraction of GSSG concentrations from TGH and a final GSH/GSSG ratio was computed. All standards and samples were run in triplicate and an average was taken. The intra- and inter-coefficient of variance for the assay kits was 3.4% and 3.6% respectively.

3.10 RNA extraction

RNA was extracted using previously validated methods (Chomczynski and Sacchi, 1987). Briefly, chloroform (Sigma Aldrich, Dorset, UK) was added to (200 µL for muscle samples; 40 µL for leukocytes samples) samples suspended in TRIzol® reagent, then vortexed and left to incubate on ice for 10 min prior to centrifugation at 17 000 G for 15 min at 4°C. The resulting sample separates into an aqueous clear phase containing RNA and chloroform; a small white interphase comprising of DNA and protein; and a large pink phase containing TRIzol and cellular remnants. The aqueous phase was carefully aspirated into a fresh 1.5 mL RNA-free eppendorf and equal volume of ice-cold propan-2-ol (Sigma Aldrich, Dorset, UK) was added before a 15 min incubation period on ice and subsequent centrifugation at 17 000 G for 15 in at 4°C. The supernatant was removed and the sample was washed with ice-cold 75% ethanol (Sigma Aldrich, Dorset, UK) (1 mL for muscle samples; 100 µL for leukocytes samples) ahead of centrifugation at 5 400 G for 8 min at 4°C. Two additional ethanol washes were performed. Remaining ethanol was aspirated and the pellet was allowed to air dry for 5 min prior to the addition of 50 µL of RNA storage solution (Invitrogen, Paisley, UK), followed by a final vortex for 90 s.

All procedures outlined above were performed using RNA-free pipette tips and pipettes that were solely used for RNA work, on surfaces and equipment which had been decontaminated with 70% industrial methylated spirit and RNase ZAP (Ambion, The RNA Company Cheshire, UK) prior to commencement of RNA

work. Fresh tips were used for each sample to avoid sample cross-contamination. The samples were frozen at -80°C for future RNA concentration quantification.

3.11 RNA concentration quantification

RNA concentrations and purity were calculated by spectrophotometry analysis utilising the Nanodrop 2000c. 2 µL of RNA storage solution was placed onto the pedestal as the blanking solution prior to sample measurement. Subsequently, 1µL of sample was placed onto the pedestal and measured at wavelengths of 260 nm and 280 nm. The ratio of 260/280 was used to assess the purity of the RNA sample, where the ratio 2.0 was considered to be "pure" RNA. Samples were considered "high quality" at ratios in the range of 1.90 – 2.10 and were accepted for use in quantitative real-time polymerase chain reaction (RT-PCR). Concentrations of RNA were calculated using a modification of the Beer-Lambert equation:

$$c\ (ng \cdot \mu L^{-1}) = \frac{A\ (AU) \times\ \varepsilon(ngcm \cdot \mu L^{-1})}{b\ (cm)}$$

Where c is the nucleic acid concentration; A is the absorbance; ε is the wavelength-dependant extinction coefficient (40 ngcm·µL^{-1} for RNA); and b is the pathlength.

3.12 One-step quantitative real-time polymerase chain reaction

RT-PCR was performed on a thermal cycler (RotorGene, Qiagen, Manchester, UK) using QuantiFast® SYBR® Green RT-PCR kits (Qiagen, Manchester, UK) containing: (2x) QuantiFast SYBR Green RT-PCR Master Mix (HotStarTaq®

plus DNA Polymerase, Quanitfast SYBR green RT-PCR buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), ROX passive reference dye) and (1x) QuanitFast reverse transriptase (RT) mix (Omniscript® RT, Sensiscript® RT).

Primers in Table 3.2 were designed by Sigma Aldrich (Dorset, UK). 20 µL of reaction mix (10 µL of SYBR green, 0.15 µL forward primer, 0.15 µL reverse primer, 0.2 µL reverse transcriptase, 9.5 µL of sample (70 $ng \cdot \mu L^{-1}$ of RNA)) was distributed using an automated pipetting machine (QiAgility, Qiagen, Manchester, UK).

The amplification program involved a preliminary denaturation phase at 50°C for 10 min followed by further holding at 95°C for 5 min. Samples then undertook 40 cycles of denaturation lasting 10 s at 95°C with a subsequent annealing and extension phase for 30 s at 60°C. SYBR green fluorescence was measured after each cycle. Melting curve analysis was then performed concluding the 40 cycles, where samples were incubated at 50°C and heated to 99°C with the fluorescence measured every 1°C increase. All samples were performed in duplicate.

Table 3.2: Primer Sequences

Target Gene	Primer Sequence (5'-3'-)	Reference Sequence Number	Amplicon Length (bp)	GC% Content
B_2 microglobulin	Forward: CCGTGTGAACCATGTGACT	NM_004048	19	52.63
	Reverse: TGCGGCATCTTCAAACCT		18	50.00
HSP 72	Forward: CGCAACGTGCTCATCTTTGA	NM_005345	20	50.00
	Reverse: TCGCTTGTTCTGGCTGATGT		20	50.00
HSP32	Forward: CAGCAACAAAGTGCAAGAT	NM_002133	19	42.11
	Reverse: CTGAGTGTAAGGACCCATC		19	52.63

3.12 Quantitative real-time polymerase chain reaction analyses

Samples displaying multiple peaks in the melting curve were excluded from further analyses. RotorGene software plotted the sample fluorescence against cycle number on a graph and the cycling threshold was manually positioned above background fluorescence levels where there was an exponential rise in fluorescence. Gene expression was determined through the ratio between the target gene and the housekeeping gene, ß$_2$-microglobulin, and was calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method (outlined by Schmittgen and Livak (2008)), where relative gene expression was determined using $2^{-\Delta\Delta CT}$.

3.13 Statistical Analyses

All data was analysed using the statistical software package IBM SPSS version 19.0 (SPSS Inc, Chicago IL, USA). Prior to any performance of inferential statistics, descriptive tables and graphical methods (Q-Q plots and scatter plots) were utilised to check for statistical assumptions. All data presented was deemed to be normally distributed. A number of outliers were observed during exploratory data analysis and were subsequently removed prior to performance of inferential statistics (n = 6 in each condition unless otherwise stated). A one-way analysis of variance (ANOVA) was used to assess for statistical differences between participants' anthropometric data. A one-way repeated measures ANOVA was utilised to establish significant differences between haemoglobin saturation and HR during the hypoxic intervention period. Sphericity was assumed for all repeated measures analysis. Linear mixed models (LMMs) were used to identify significant group x time interactions in the remaining dependant variables across all groups. In the event of a significant F ratio for both LMMs and one-way repeated measures ANOVAs, the post-hoc test Sidak was used to locate significant pairs. LMMs were chosen as this particular type of statistical analyses allows for missing data, for non-independent data and the best appropriate covariant structure to be selected (Field, 2009). The most suitable covariant model was decided using the difference in -2 restricted log likelihood figures and the number of parameters of the two models tested against the χ^2 critical statistic (Field, 2009). Furthermore, residuals were checked for normality and homogeneity of variance using Q-Q plots and scatter plots respectively, and were considered plausible for all dependant variables. Statistical significance was

assumed at $p < 0.05$. Finally, Cohen's effect sizes (ESs) for independent means were calculated utilising the formula outlined by Cohen (1992):

$$d = \frac{\mu_a - \mu_b}{\sigma}$$

The quantity *d* is the standardised mean difference, where μ_a and μ_b are separate means. The value σ is the pooled standardiser and is computed using the subsequent equation described by Olejnik and Algina (2000):

$$\sigma = \sqrt{\frac{(n_a - 1)SD_a{}^2 + (n_b - 1)SD_b{}^2}{(n_a - 1) + (n_b - 1)}}$$

The quantities n_a, SD_a and n_b, SD_b represent the sample size and SD for μ_a and μ_b respectively. The ES was established as: small ($d = 0.2$), medium ($d = 0.5$) and large ($d = 0.8$) effects (Cohen, 1992).

Chapter 4: Results

No significant differences ($p \geq 0.34$) in participant demographics were observed between SHAM, HYP or TOR, with the exception of age ($F_{2,15} = 4.36$, $p = 0.32$) noted between conditions TOR and SHAM ($p = 0.032$) (Table 3.1).

A significant main effect displayed a decrease in haemoglobin saturation ($F_{8,40} = 17.331$, $p < 0.001$) between baseline and all subsequent time points ($p < 0.05$) in the HPC intervention experienced by HYP. However, there was no significant main effect ($F_{8,40} = 1.130$, $p = 0.365$) in HR noted by the same exposure (Figure 4.1).

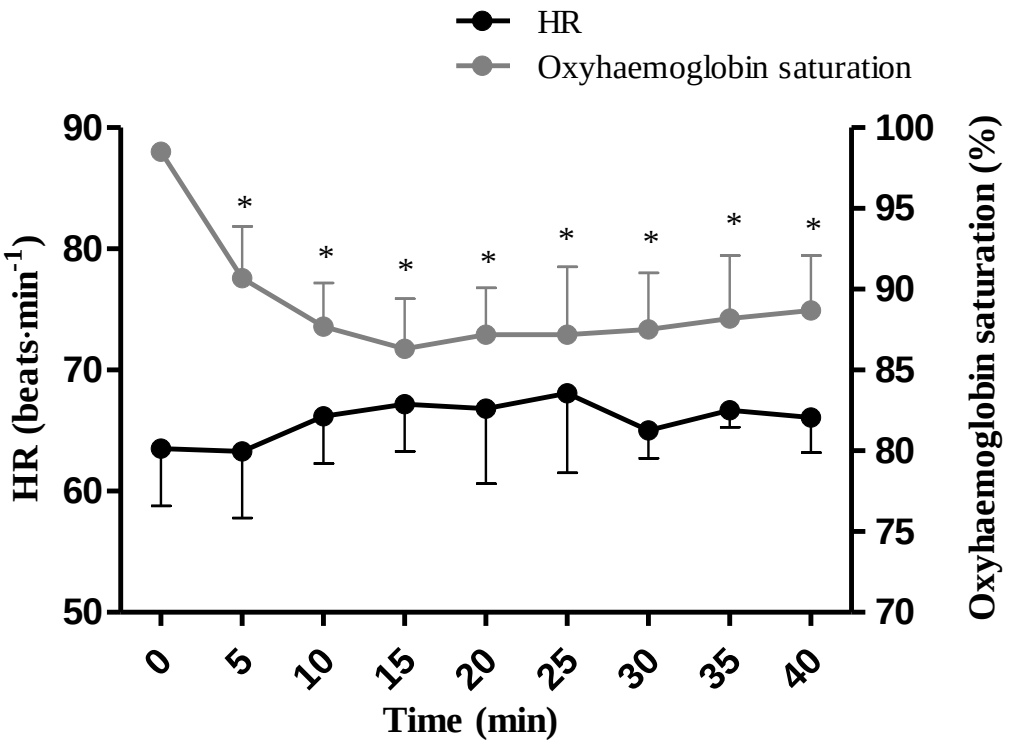

Figure 4.1: Mean HR and oxyhaemoglobin saturation during HYP intervention. * indicates significant difference v.s. baseline value. Error bars represent SD. Abbreviations: HR – heart rate

4.1 Circulatory stress and redox markers

There were no significant ($p > 0.05$) group x time interaction effects for leukocyte Hsp72 ($F = 1.195$, $p = 0.347$), leukocyte Hsp32 ($F = 1.406$, $p = 0.244$), PC ($F = 0.681$, $p = 0.707$), TGH ($F = 0.510$, $p = 0.844$), GSSG ($F = 0.510$, $p = 0.844$), GSH ($F = 0.856$, $p = 0.564$) or GSH/GSSG ($F = 1.959$, $p = 0.105$) (Table 4.1).

Table 4.1: Mean (SD) systemic circulatory stress and redox markers across basal, immediately post-intervention (PoI), immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT) and 120 min post-tourniquet removal (120PoT)

Measure	Basal			PoI			PrT			15PoT			120PoT		
	SHAM	**HYP**	**TOR**	**SHAM**	**HYP**	**TOR**	**SHAM**	**HYP**	**TOR**	**SHAM**	**HYP**	**TOR**	**SHAM**	**HYP**	**TOR**
Hsp72 (relative fold change from basal)	1.46 (0.42)	1.29 (0.39)	1.72 (0.54)	1.46 (0.55)	1.23 (0.44)	1.31 (0.17)	1.43 (0.41)	1.45 (0.36)	1.31 (0.35)	1.44 (0.43)	1.57 (0.31)	1.33 (0.14)	1.23 (0.44)	1.35 (0.27)	1.35 (0.21)
Hsp32 (relative fold change from basal)	1.34 (0.43)	1.08 (0.24)	1.47 (0.55)	1.09 (0.33)	1.14 (0.24)	1.23 (0.40)	0.86 (0.14)	1.10 (0.28)	1.29 (0.29)	0.86 (0.28)	1.24 (0.27)	1.17 (0.33)	1.04 (0.45)	1.02 (0.41)	1.08 (0.38)
Protein Carbonyl ($nmol \cdot mL^{-1}$)	0.56 (0.14)	0.56 (0.19)	0.69 (0.24)	0.54 (0.16)	0.42 (0.13)	0.61 (0.13)	0.64 (0.13)	0.58 (0.08)	0.69 (0.21)	0.65 (0.18)	0.60 (0.24)	0.56 (0.15)	0.65 (0.32)	0.69 (0.15)	0.63 (0.21)
Oxidised glutathione (pmol)	190 (87)	155 (70)	177 (96)	178 (70)	135 (34)	140 (35)	166 (52)	150 (63)	216 (127)	174 (50)	216 (56)	173 (69)	205 (83)	202 (75)	208 (97)
Reduced glutathione (pmol)	3815 (603)	4441 (598)	4081 (607)	3874 (653)	5218 (895)	4431 (520)	4021 (557)	4256 (998)	4397 (614)	4386 (366)	4163 (720)	4389 (864)	4224 (390)	4280 (585)	4353 (870)
Reduced/oxidised glutathione ratio	23.0 (9.1)	31.4 (12.2)	22.7 (8.6)	24.6 (9.6)	29.3 (5.4)	28.9 (7.4)	27.1 (11.6)	23.9 (7.3)	18.6 (7.6)	27.6 (10.7)	20.5 (3.6)	23.1 (8.7)	23.0 (7.7)	22.4 (2.2)	20.5 (9.6)
Total glutathione (pmol)	4006 (646)	4596 (650)	4258 (580)	4052 (649)	4352 (936)	4434 (561)	4187 (532)	4358 (888)	4596 (555)	4374 (557)	4343 (797)	4563 (828)	4395 (462)	4488 (580)	4625 (399)

Hsp – heat shock protein gene expression

4.2 Localised muscle stress markers

Hsp72 gene expression

Significant group x time interaction effects (F = 3.058, p = 0.048) were observed in muscle Hsp72 relative gene expression. There was a 76% mean increase between PrT and 15PoT (95% CI -3.771, -0.124; p = 0.035) in SHAM displaying a large ES (1.44). Also, a pronounced 116% increase between PrT and 120PoT (95% CI -3.779, -0.400; p = 0.014) was noted in TOR also producing a large ES (1.59). Furthermore, there was a 51% and 50% decrease in HYP (95% CI 0.634, 3.934; p = 0.007) and TOR (95% CI 0.675, 4.114; p = 0.006) respectively when compared to SHAM at 15PoT both demonstrating large ESs (1.90 and 2.19 respectively) (Figure 4.2). (SHAM, n = 5; HYP, n = 6; TOR, n = 6).

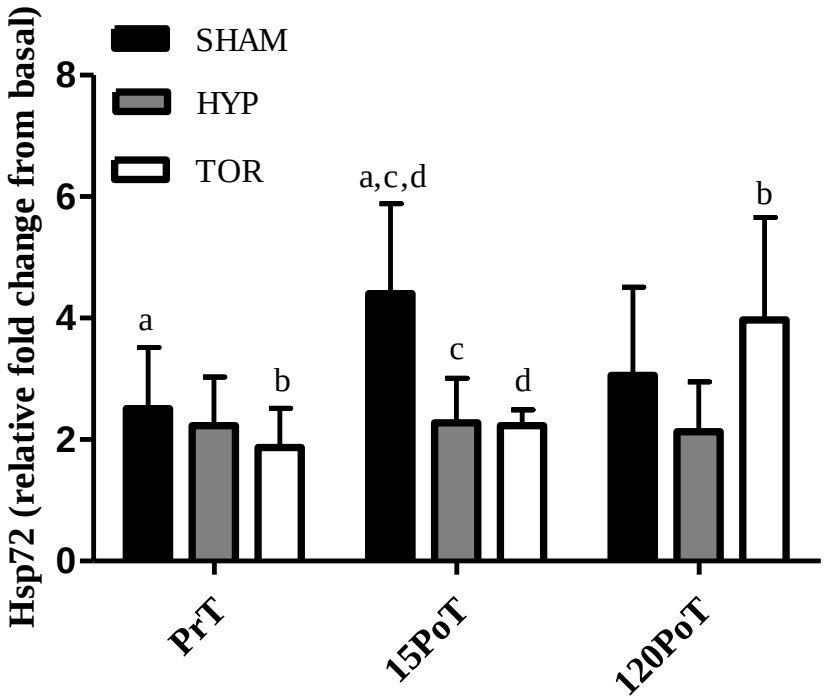

Figure 4.2: Mean muscle Hsp72 relative gene expression at immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT) and 120 min post-tourniquet removal (120PoT) in all conditions. Like letters denote significant differences (p < 0.05) between mean values. Error bars represent SD.

Hsp32 gene expression

There were no significant group x time interactions (F = 0.147, p = 0.961) in muscle Hsp32 gene expression over PrT, 15PoT and 120PoT (Figure 4.3) (SHAM, n = 5; HYP, n = 6; TOR, n = 6).

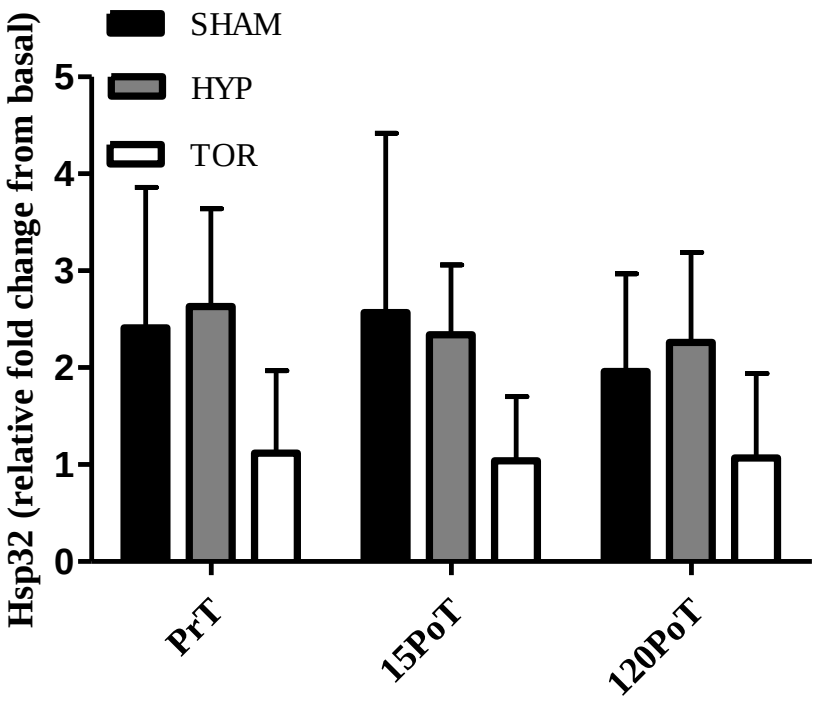

Figure 4.3: Mean muscle Hsp32 relative gene expression at immediately pre-tourniquet application (PrT), 15 min post-tourniquet removal (15PoT) and 120 min post-tourniquet removal (120PoT) in all conditions. No significant interaction effects were noted at any time point Error bars represent SD.

Chapter 5: Discussion

The purpose of the present study was to examine whether HPC and IPC elicited a reduction in oxidative stress to knee surgery specific tourniquet application. Furthermore, to elucidate a time-course for circulatory oxidative stress markers (GSH, GSSG, GSH/GSGG ratio, PC); in addition to circulatory (leukocyte) and localised (muscle) stress protein expression (Hsp72 and Hsp32). In contrary to the hypothesis, the results revealed that a bout of either HPC or IPC did not produce a statistically significant reduction in systemic Hsps, redox stress markers, or in localised Hsp32 in comparison to control. However, both HPC and IPC did demonstrate a significant reduction in Hsp72 at 15PoT in the localised gastrocnemius tissue from TKR specific tourniquet application.

5.1 Circulatory redox and stress markers

Interestingly, the intervention groups did not display a significant difference in oxidative stress markers in comparison to the control following TKR specific tourniquet application. This is in disagreement with Koca et al. (2011) who observed stable redox markers (malondialdehyde (MDA), total oxidant and antioxidant capacity) in the IPC group in comparison to a significant negative response noted in control following knee arthroscopy surgery. However, the authors failed to implement any subject dietary restrictions, which could have markedly affected the subjects' antioxidant capacity (Powers et al., 2010b) and could potentially have impacted upon the results published. Furthermore, smoking has been shown to increase ROS production (Kalra et al., 1991), however, Koca and colleagues (2011) did not exclude smokers from their research, thus the experimental findings displayed may not be a true representation of their

experimental manipulation. Finally, the measure of MDA via thiobarbituric acid assay utilised by Koca et al. (2011) is considered to be an unacceptable measure, as the majority of thiobarbituric acid material found in the body is not related to MDA (Powers et al., 2010b), perhaps explaining the differing redox results between the current study.

It could be speculated that the 30 min bout of limb ischemia utilised here did not induce a sufficient level of stress to observe the hypothesised potential favourable effects bestowed from the preconditioning interventions in the systemic blood. Previous surgical literature demonstrating the beneficial effects of IPC through circulatory markers implemented a far greater ischemic periods (mean ± SD; 89 ± 9 min (Koca et al., 2011)). This extended ischemic period would induce greater quantities of activated leukocytes and ROS into the circulation, thus stimulating further cellular structure oxidation. Therefore, without the initial systemic oxidative burst, preconditioning would not provide a noticeable benefit. Indeed, this hypothesis is reinforced via the stable leukocyte stress response (Hsp) data depicted in Table 4.1.

HSPs are up-regulated during a variety stressors, such as oxidative stress, hypoxia and ischemia (Morton et al., 2009). The homeostatic insults lead to protein denaturation and unfolding, initiating the heat shock response, thus permitting heat shock factor 1 (HSF1) to oligermerise and bind to the heat shock element promoting gene transcription (Noble et al., 2008). HSP72 provides cytoprotection through refolding of denatured proteins and rescuing apoptotic cells via interruption of the programmed death cascade (Taylor et al., 2011). An increase in

Hsp72 would demonstrate greater cellular stress (Theodorakis et al., 1999), however, as aforementioned; no significant increases in leukocyte Hsp72 were noted in peripheral blood in any of the conditions following the TKR specific tourniquet application (Table 4.1). Therefore, it could be inferred that there was no systemic stress following 30 min limb ischemia.

Surprisingly, there were also no significant changes in leukocyte Hsp72 concentration at PoI in HYP or TOR following the preconditioning (Table 4.1). A rise in Hsp72 following HPC or IPC would be expected as the initiation of both interventions invokes an oxidative burst (Konstantinov et al., 2004; Taylor et al., 2010), thus stimulating the heat shock response. In contrast to the results in the current study, Konstantinov et al. (2004) showed an increase in peripheral leukocyte Hsp72 using microarray analyses following IPC in the forearm. Although the authors observed a rise in leukocyte Hsp72, there is evidence to suggest that microarray analyses can overestimate gene expression (Feldman et al., 2002). Furthermore, Taylor et al. (2010) noted a significant increase in Hsp72 following an acute hypoxic exposure, which again is in contrary to the data following HPC (Table 4.1). The disparity between Taylor et al. (2010) and the present study may be due to the longer hypoxic period utilised by the authors, thus providing greater systemic stress to induce a larger up-regulation in Hsp72.

Both Taylor et al. (2010) and Konstantinov et al. (2004) used different techniques (flowcytometry and microarray respectively) of quantifying the changes in stress protein response in comparison to the present study (RT-PCR). However, measurement of gene expression via RT-PCR is considered to be a sensitive and

reproducible method in which to quantify gene expression (Wong and Medrano, 2005), as such, would be able to identify the appearance of small changes in gene expression.

A rise in leukocyte Hsp32 would be anticipated following an oxidative stress insult (Fehrenbach et al., 2003). The rapid induction of HSP32 has been proposed to stimulate protection via the catabolism of the reactive free heme into carbon monoxide and biliverdin (Gozzelino et al., 2010). However, the present study did not observe a significant increase in leukocyte Hsp32 (Table 4.1). Despite the evidence depicting the vital role HSP32 plays in counteracting oxidative stress (Gozzelino et al., 2010), no previous research has assessed Hsp32 with regards to human limb ischemia *in-vivo*. Therefore, this finding (stable systemic Hsp32 concentrations following preconditioning and limb ischemia) can be considered novel.

Glutathione concentrations have been previously measured in the systemic blood following TKR surgery (Mathru et al., 1996; Karg et al., 1997). Interestingly, both Karg et al. (1997) and Mathru et al. (1996) only observed changes in GSSG immediately following reperfusion in the localised blood supply rather than the systemic circulation. The observation of stable systemic glutathione markers is in accordance with the present study's findings (Table 4.1). This would suggest that the localised circulation offered protection to the remote blood supply from potentially damaging ROS. Indeed, it has been shown that intact erythrocytes scavenge H_2O_2, providing vital protection to distant organs (Toth et al., 1984). The non-significant change in systemic glutathione markers also demonstrates the

safe duration of at least 30 min limb occlusion, as previous research has associated prolonged limbed ischemia (3 hrs) with multiple organ dysfunction syndrome via circulating ROS and activated leukocytes (Yassin et al., 2002).

The assessment of PC concentration in the blood following knee specific tourniquet mediated ischemia is a novel finding. In fact, the majority of studies assessing limb occlusion have evaluated PC concentrations in the localised tissue (Ozyurt et al., 2006; Avci et al., 2012; Ozkan et al., 2012). In contrast to the data provided here, previous studies assessing plasma PC concentrations following ischemia-reperfusion in other tissues (cardiac, intestinal) displayed a significant increase in plasma oxidised protein concentrations (Narayani et al., 2003). It could be speculated that different sampling sites (i.e. systemically or directly from the ischemic site) and varying experimental durations of ischemia-reperfusion utilised by Narayani et al. (2003) could account for the disparity in plasma PC concentrations in comparison to the current study. However, the authors failed to state this information, creating difficulties in producing valid comparisons to the work presented here.

Furthermore, the stable concentrations of PC observed here (Table 4.1) are not surprising considering the lack of significant variation in the GSSG concentrations. Indeed, the simultaneous increase in both GSSG and PC concentrations has previously been noted following ischemia reperfusion in rat hindlimb (Grisotto et al., 2000). The ischemic bout promotes the formation of ROS (such as $OH^{\bullet}$, O_2^-) and ROS associated intermediates (H_2O_2) upon reperfusion, inducing cellular protein oxidation. GSH metabolises the ischemic

mediated H_2O_2, thus removing the intermediate in the chain reaction that synthesises the extremely reactive $OH^{\bullet}$, invariably forming GSSG and minimising protein damage (Mari et al., 2013). Thus, a fairly constant GSH/GSSG ratio indicates minimal disruption to the redox balance (Asensi et al., 1999), therefore, negligible protein oxidation would occur.

5.2 Muscle HSP expression

The blunting of the tourniquet induced response noted by the reduction in Hsp72 displayed in both HYP and TOR in comparison to SHAM at 15PoT, also showed a large effect size (1.90 and 2.19, respectively) (Figure 4.2). Similar observations were noted by Bushell et al. (2002). The authors showed IPC did not stimulate an increase in skeletal muscle Hsp72, when a dramatic rise was observed in the control condition. Interestingly, the authors still witnessed protection in the IPC tissue following an ischemic insult, in spite of the stable Hsp72 concentrations, thus, indicating that perhaps HSP72 does not play a role in early preconditioning. Therefore, the blunted response observed in both TOR and HYP in comparison to SHAM would suggest a reduction in cellular stress from conditioning (Theodorakis et al., 1999) rather than an IPC mechanism.

IPC acts in a biphasic pattern, with the early phase of protection being initiated immediately post preconditioning and lasting up to 3 hrs (Yang et al., 2010); while the delayed phase occurs 24 hrs following the stimulus, enduring for up to 72 hrs (Hausenloy and Yellon, 2010). Previous research implicates adenosine as a key molecule in initiating early IPC (Liu et al., 1991). The ischemic environment leads to the degradation of AMP via 5'-nucleotidase (Kitakaze et al., 1995),

directly stimulating protein kinase C (PKC) (Carden and Granger, 2000). PKC has been proposed to act upon the mitochondrial potassium ATP (mK_{ATP}) channel and the mitochondrial permeability transition pore (mPTP), with the former inducing an influx of K^+, further stimulating PKC (Sadat, 2009). The activation of PKC inhibits the mPTP, whilst also activating cytochrome C oxidase, further increasing cellular respiration, thus protecting the cell from excessive ROS (Sadat, 2009). Besides adenosine, bradykinin can also stimulate PKC indirectly through ERK and redox signalling, ultimately inducing PKC activation via ROS stimulation (Cohen et al., 2007).

Although the mechanism described above explains the diminished tourniquet mediated stress in TOR, it does not directly provide evidence for the same Hsp72 decrease noted in HYP. However, it has been previously stated that HPC acts through similar redox mechanisms as IPC (Zuo et al., 2013). In fact, this would be logical considering the increase in adenosine is produced via the degradation of AMP (Kitakaze et al., 1995) that has accumulated through the disturbance of aerobic respiration (Jennings and Reimer, 1991), which can be produced by both hypoxia and ischemia. This has been confirmed via pharmaceutical blockade of PKC, the mK_{ATP} channel and adenosine receptors, ultimately abolishing the protective effects of HPC in cardiomyocytes (Nojiri et al., 1999). Interestingly, the same study noted that nicorandil, a mK_{ATP} channel opener, which has previously been shown to induce HPC protection from IRI, did not bestow the same protection when PKC was inhibited, demonstrating that mK_{ATP} channel opening leads to PKC activation, which is in contrast to the activation mechanism of IPC. Therefore, the reduction in ischemic mediated stress observed in both

TOR and HYP in the present study (Figure 4.2) could be produced via the aforementioned mechanism.

A significant increase in Hsp72 at 120PoT compared to PrT was noted in TOR (Figure 4.2). Although not in skeletal muscle, a similar rise in Hsp72 was cited in rabbit cardiac tissue 3 hrs following IPC (Tanaka et al., 1998). Previous research has described HSP72 as a major instigator in producing the delayed effects of IPC to afford protection for future insults (Lepore et al., 2000; Okubo et al., 2001; Li et al., 2003). Consequently, the sharp rise in Hsp72 at 120PoT displayed by TOR could potentially be explained by the delayed phase of IPC. However, the elevation in Hsp72 noted here is following the combination of both IPC and TKR specific tourniquet application, not just following IPC as mentioned by previous research (Tanaka et al., 1998; Li et al., 2003), hence it is not classical delayed IPC. Thus, it could be suggested that the rise in Hsp72 could merely be a result of oxidative stress from the TKR specific tourniquet application (Lepore et al., 2001); nonetheless if this were the case, a similar increase would have been expected in SHAM (Figure 4.2). Therefore, it could be inferred that the presence of increased Hsp72 concentrations could be explained by the delayed preconditioning phenomenon from the IPC.

The postponed protection afforded by IPC has been attributed to *de novo* synthesis of cytoprotective proteins such as HSPs, eNOS, cyclooxygenase-2 and MnSOD (Hausenloy and Yellon, 2010). The up-regulation of these proteins occurs through the activation of a multitude of transcription factors, stimulated by endogenous mediators including PKC, adenosine and tyrosine kinase (Heusch et al., 2008; Yin

et al., 2009). The synthesis of HSP70 is speculated to refold sub-lethal damaged proteins or diminish their interactions with viable proteins during the prolonged ischemia/reperfusion bout (Marber et al., 1993). This is further supported by evidence of infarct reduction in rabbit models overexpressing HSP72 (Okubo et al., 2001). In addition to providing cytoprotection, HSP70 has also been shown to mediate κ-opioid receptor stimulation, which are in part responsible for delayed IPC (Zhou et al., 2001). This may partly explain the increase in Hsp72 at 120PoT depicted in Figure 4.2.

In contrary to the research that considers HSP72 crucial to delayed IPC, it has also been proposed that the occurrence of HSP72 24 hrs following IPC may in fact be a marker of delayed preconditioning, rather than the mechanism involved in providing protection (Pagliaro et al., 2001). Qian et al. (1999) demonstrated a marked increase in HSP72 24 hrs following IPC in rat myocardium, however, this rise did not induce protection in myocardial tissue. The authors concluded that the discrepancy could be a variation in species response to delayed preconditioning. Nevertheless, it has been proposed that PKC may also play a pivotal role in both phases of preconditioning, with the activation of PKC in the delayed phase governed by tyrosine kinase and NO, ultimately inducing further opening of the mK_{ATP} channel (Pagliaro et al., 2001). However, recent novel research has instigated the small non-coding microRNAs as another potential mechanism of delayed IPC (Yin et al., 2009). Yin and colleagues (2009) demonstrated that injection of microRNAs reduced the infarct size in murine hearts and up-regulated HSP70, eNOS and hypoxia induced factor -1α, possibly through post-transcriptional regulation of injurious genes. The rise in Hsp72 noted in the

current research (Figure 4.2) could potentially be a precursor to delayed preconditioning; however the literature remains controversial regarding this hypothesis.

Surprisingly, HYP did not display the dramatic rise in Hsp72 demonstrated by TOR at 120PoT. This is unexpected considering the evidence outlined above depicts that both HPC and IPC appear to share similar molecular mechanisms. However, it could be hypothesised that HYP demonstrated a severe reduction in oxidative stress during the TKR specific tourniquet application compared to TOR, thus leading to reduced HSP response via hypoxia induced Hsp72 down-regulation (Oehler et al., 2000). Furthermore, the negligible elevation in Hsp72 at 120PoT would also indicate the absence of HSP72 in inducing delayed preconditioning as aforementioned in HYP. This is in disagreement with previous literature, which has attributed HSP72 in producing the delayed preconditioning phase (Engelman et al., 1995). Nevertheless, the study by Engelman et al. (1995) displayed a rise in Hsp70 4 hrs following a prolonged hypoxic exposure post HPC, thus it could be speculated that the stable Hsp72 demonstrated here (Figure 4.2) was merely a disparity in sampling time and may occur later than IPC. Interestingly, the same study did not find an increase in Hsp70 ensuing HPC prior to the sustained hypoxic insult. This is in line with the current study where no significant difference was observed between HYP and SHAM at PrT. It could be suggested that the absence of an increase in Hsp72 following HPC could potentially be via the inhibition of the kinase, mammalian target of rapamycin (mTOR). mTOR has been cited as a major kinase involved with crucial phosphorylation of HSF1 following dissociation from HSPs (Chou et al., 2012).

However, acute normobaric hypoxia has been demonstrated to inhibit mTOR function (D'Hulst et al., 2013), thus preventing HSF1 phosphorylation and further transcription of Hsp72. This mechanism may explain the stable concentrations of Hsp72 (Figure 4.2) observed in the current research.

The present study did not show a change in muscle Hsp32 between any of the conditions (Figure 4.3). This is in disagreement with previous research showing that Hsp32 expression transiently increased following HPC (Berger et al., 2010) and greater protein expression succeeding IPC (Badhwar et al., 2004). It is postulated that the lack of variation in Hsp32 concentrations could be via Hsp32 transcriptional repression during the hypoxic insult. Nakayama et al. (2000) established that heme oxygenase-1 (HSP32) is down-regulated in ECs during the hypoxic exposure. It has been postulated that the reduction in Hsp32 concentrations could consequently reduce the large energy expenditure associated with heme degradation, preserving vital ATP stores (Nakayama et al., 2000). Additionally, the reduction in translation of *de novo* HSP32 during the hypoxic period would diminish the immediate production of CO, preventing its binding to oxygen sensing heme molecules, invariably disrupting their function (Shibahara, 2003). Indeed, the transcription factor Bach-1 has been identified as a regulator of HSP32 (Ogawa et al., 2002). The presence of heme negatively affects Bach-1's ability to repress HSP32 gene expression via DNA binding (Ogawa et al., 2002).

The present data did not show a significant down-regulation of muscle Hsp32 throughout the assessed time-points, nevertheless, this could possibly be due to the short hypoxic exposure (40 min hypoxia, 60 min recovery, 30 min ischemia)

experienced by the participants, thus resulting in the equalised Hsp32 concentrations observed over the experimental period (Figure 4.3). It could also be speculated that the stable concentrations of Hsp32 may be a fibre type specific response. Type I muscle fibres have been shown to readily express HSP32 in comparison to a blunted response noted in type II fibres (Vesely et al., 1999). The lateral head of the gastrocnemius consists of equal proportions of both fibre types (Edgerton et al., 1975), therefore the response observed in the present study may only be proportional to the percentage of type II fibres in the muscle.

5.3 Application of results

Recently, the use of IPC as non-pharmaceutical, non-invasive intervention has been cited to diminish postoperative pain following TKR surgery (Memtsoudis et al., 2010). Although the difference in pain noted by Memtsoudis and colleagues (2010) was not the main aim of the study, the simplicity of IPC allowed it to be performed during draping/surgical preparation ensuring no delay to commencement of surgery, thus, even a modest improvement in patient perceived pain should be seen as a positive and an advocate for the use of IPC as an addition during TKR surgery. The present study has also demonstrated that HPC provides similar protective effects to IPC against 30 min tourniquet mediated ischemia. In addition, the simplicity of the HPC protocol used here could also be undertaken during the pre-operative phase while the patient is still on the ward. The present HPC protocol has the added benefit of less staff involvement than IPC through redundancy of tourniquet inflation/deflation monitoring.

Furthermore, length of stay has been demonstrated to significantly decrease following IPC in comparison to control in TKR out-patients (Memtsoudis et al., 2010). Although these results (reduction in pain and LOS) are promising, the study itself was not specifically designed to identify clinical outcomes; therefore further research in this area is warranted. The similar effects induced by protection bestowed from HPC and IPC in the current study could potentially reduce length of stay comparatively to Memtsoudis et al. (2010) through attenuation of muscular tissue damage mediated through tourniquet use. Thus, if this were confirmed in a study designed to solely address this entity, it could potentially have a large effect upon clinical practise in the future.

5.4 Limitations

Firstly, many antioxidants are procured naturally from the diet, all with varying half-lives, potentially influencing the antioxidant defence capacity of the individual (Powers et al., 2010a). Although every effort was made to minimise this (standardised evening, morning and afternoon meal), it is extremely challenging to control participants' diet over a long period of time, while ensuring continued participation. Therefore, the lack of change in oxidative stress markers could have partly been due to dietary variation.

Secondly, difficulties obtaining blood samples from the site of ischemia (i.e. the right calf) hindered the possibility of further inferences from this data. If samples were collected from the ischemic site, the data may have alluded to a greater insight into the potential link between the localised tissue and the systemic blood. Previous research (Karg et al., 1997; Garcia-de-la-Asuncion et al., 2012) has

shown disparity between markers at the systemic and localised sites, consequently the oxidative stress markers measured in the present study may not depict the full cascade of events.

Finally, the duration of limb ischemia utilised in this study (30 min) is lower than commonly used during TKR surgery (mean ± SD; 79.9 ± 12.7 min (Chang et al., 2012)). Therefore, it is expected that the oxidative stress would be greater during surgery thus the current model performed in the study is not completely ecologically valid.

5.5 Conclusions

Overall, systemic markers of oxidative stress did not change during the trial in any condition, thus producing a stable time course for redox (GSH, GSSG, TGH, GSH/GSSG, PC) and stress protein markers (Hsp72, Hsp32) following both preconditioning and TKR specific tourniquet application. In addition, HPC and IPC did not induce a marked reduction in the systemic oxidative stress measures compared with a control condition. However, the significant reduction in localised cellular stress noted in both TOR and HYP at 15PoT is encouraging. This provides further evidence for protection offered via IPC but also demonstrates the potential of HPC in diminishing cellular stress associated with TKR specific tourniquet application, although the precise mechanisms of action were not alluded to in this study. The diminished localised stress provides a rationale for the extension of this research into a clinical population to establish more clinically-relevant qualitative measures for patient's perceived pain and levels of surgical success.

Finally, to conclude this thesis, it is appropriate to revisit the experimental aims described at the end of the literature review section. The aims are restated below and an appropriate answer has been provided.

1) Quantify the time course for redox disturbances to the systemic and localised circulation via analysis of PC, GSH, GSSG and TGH, following hypoxic and ischemic preconditioning, in addition to 15 min and 2 hrs succeeding tourniquet mediated ischemia.

   - Systemic circulation concentrations of PC, GSH, GSSG and TGH did not significantly fluctuate from basal following any of the interventions (SHAM, HPC, and IPC) and TKR specific tourniquet application. Localised circulatory redox markers were not assessed due to problems with localised blood collection.

2) Examine the time course for changes in Hsp72 and Hsp32 in localised skeletal muscle, in addition to localised and systemic leukocytes utilising the same time points as outlined in 1).

   - HPC and IPC displayed a blunted Hsp72 response in skeletal muscle in comparison to SHAM 15 min following tourniquet release. Additionally, IPC displayed sharp increase 2 hrs post tourniquet release. Tissue Hsp32 and Systemic circulatory Hsp72 and Hsp32 did not show any alterations following tourniquet release.

3) Evaluate the efficacy of both whole-body HPC and limb IPC based on the observed changes in 1) and 2) from TKR specific tourniquet application.

   - HPC and IPC displayed a blunted Hsp72 response in comparison to SHAM 15 min following tourniquet release in skeletal muscle. Therefore,

suggesting that both HPC and IPC provided cellular protection to tourniquet mediated oxidative in localised tissue.

5.6 Recommendation for future research

Completion of this thesis has generated several potential areas for future research, specifically, establishment of oxidative stress markers and cell viability in the localised muscle. Furthermore, the potential benefits of preconditioning should be confirmed within a small clinical population undergoing TKR to establish more clinically relevant measures (i.e. length of stay, Oxford Knee Score, patient perceived levels of pain). The following section will provide a brief summary of studies addressing these issues.

5.6.1 Determination of redox disturbance and cellular structure in muscle tissue following HPC in a TKR specific tourniquet application

The confirmation of reduced cellular structural damage and minimised disturbance to muscle redox balance would provide empirical evidence to the effectiveness of HPC. In addition to the markers utilised here (GSH/GSSG, PC) antioxidant enzymes (e.g. MnSOD) and assessment of cell viability would provide a greater representation of the biochemical events occurring in the localised tissue following tourniquet application. An additional muscle biopsy would be added at 24 hrs post tourniquet removal to assess changes peak changes in MnSOD levels (Hoshida et al., 1993). Venous blood would also be obtained at biopsy sampling times from the femoral vein to measure redox disturbances and ROS via spin trapping (Villamena and Zweier, 2004) (Figure 5.1). The use of western blot

analysis and ELISA assay kits would confirm protein and thiol concentrations respectively.

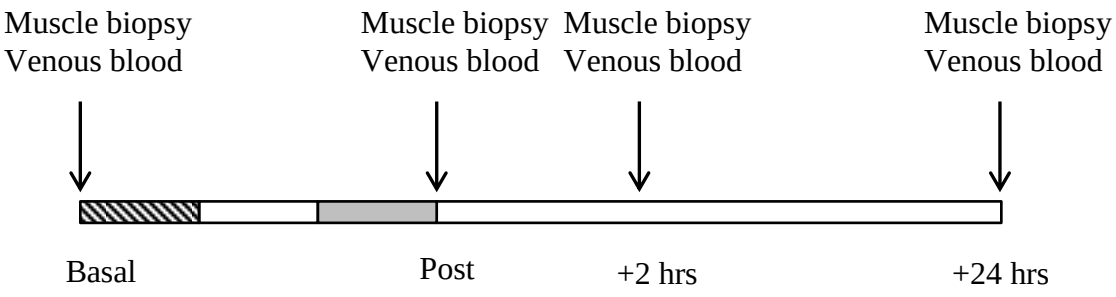

Figure 5.1: Schematic of proposed experimental design. The shaded and blocked areas represent HPC and tourniquet ischemia respectively.

5.6.2 The feasibility and clinical relevance of HPC in TKR surgery - A small clinical trial

The research would be performed in hospital and adhere to a standard operative day. Participants would be screened for inclusion criteria (bleeding disorders, immunocompromised, respiratory disorders etc.) (Memtsoudis et al. 2013) and undergo the HPC intervention while on the ward before admittance to surgery. In addition, pain (Visual analogue scale), analgesia consumption and muscular blood oxygenation would also be assessed at baseline, 6, 24 and 48 hrs post-operation (Figure 5.2). Assessment of standard TKR success criteria (Oxford Knee Scores, physiotherapy milestones) would also be recorded. These data would test the feasibility of utilising HPC in a hospital environment and provide clinically relevant information to the effectiveness of the preconditioning succeeding surgery.

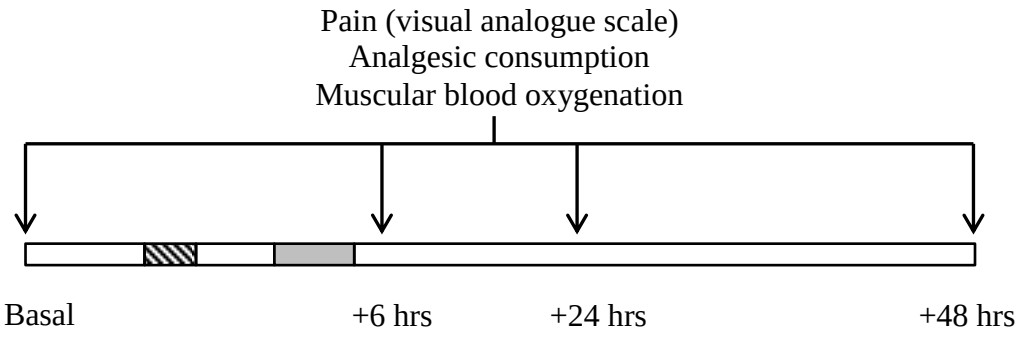

Figure 5.2: Experimental design for the clinical trial. Shaded and blocked areas represent HPC and TKR surgery respectively. Upon admittance variables would be obtained and patients would be prepared for surgery prior to commencing HPC. Succeeding surgery all measures would be collected while the patient is on the ward.

Chapter 6: References

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Appendices

Appendix A

Abstinence Criteria

Participant ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Alchol (72 hours)																								
Caffeine (72 hours)																								
Hot bath/sauna (48 hours)																								
Antioxidants (30 days)																								
Beta Alanine (15 weeks)																								
Glutathione (4 weeks)																								
Creatine (30 days)																								
Residing at Altitude (3 months)																								
Hyperthermic environments (3 months)																								

Appendix B

Information Sheet

Department of Sport and Exercise Sciences
Bedford Campus
Polhill Avenue
Bedford
MK41 9EA

Dear Participant,

Thank you for showing an interest in participating in this novel research. Please read this information sheet carefully before deciding whether to participate. If you volunteer we thank you for your participation. If you decide not to take part there will be no disadvantage to you of any kind and we thank you for considering the request.

What is the aim of the project?

The purpose of the study is to establish whether a hypoxic (low O_2 levels) exposure prior to knee surgery specific tourniquet application, provides protection against tissue damage within the subjected leg.

What type of participant is required?

Participants must be 18-35 years of age, male, and in good health.

What will the participant be required to do?

Participants will be required to attend the Sport Science Laboratories at the University of Bedfordshire's Polhill Campus on 2 occasions. You will be required to wear shorts, and abstain from alcohol and caffeine for 72 hours prior to testing. In addition you will be required abstain from intensive exercise and thermal events (sauna/very hot baths (normal bath/shower temperatures are acceptable)). Further you will be required to abstain from antioxidants, beta-alanine, creatine and glutamine for 30 days, 15 weeks, 30 days and 4 weeks respectively. Finally, please ensure you have not resided at altitude or hot environments for the previous 3 months. On the day of testing, please consume 500 mL of water 1 hr prior to start of testing to ensure adequate hydration.

Prior to testing you should complete a medical questionnaire, a blood analysis participant screening form and informed consent form, to demonstrate that you are physically capable of participating and that you understand what you are taking part in and the reasons why.

Baseline Testing
Prior to experimental testing you will visit the University of Bedfordshire's laboratories once for baseline testing. This visit will require you to have your blood pressure, height, weight and body composition measured via use of the bodpod for the later, which will last around 30-40 minutes. Following this you will be allocated either to control, sham, hypoxic, or ischaemic conditions. Although subjects will not be informed which condition they have been placed in.

Testing Overview
Control condition:
Seated in a laboratory room for 40 mins.

Sham Condition:
Seated in a laboratory inhaling normal ambient air (20.9% O_2) through a mask attached to the Hypoxicator for 40 mins

Hypoxic Condition:
Seated in a laboratory inhaling hypoxic air (14.5% O_2) through a mask attached to the Hypoxicator for 40 mins.

Ischaemic Condition:
Seated in a laboratory and have a tourniquet placed around their non-dominant thigh. Followed by 4 compression and release cycles lasting 5 minutes each. The tourniquet will be inflated to 100 mmHg above resting systolic blood pressure.

Experimental Trial
All subjects will be required to attend the laboratories at 11:00 am on their day of testing. Upon arrival you will be required to provide a urinal sample to assess hydration status. If dehydrated, subjects will be asked to drink 500 ml of water. Participants will then be asked to sit on a massage couch, during which time, two cannulae will be placed in the antecubital region (lower arm) and the small sephinous vein (calf) by either a suitably trained affiliate of the University of Bedfordshire or experienced medical practitioner from Milton Keynes General Hospital (MKGH). Subjects will then be requested to sit and rest for 1 hour. A blood sample from both cannulae will be obtained to assess levels of fold change in heat shock protein (HSP) 72 and 32, in addition to determining protein and oxidative stress concentrations.

Participants will then place, on to themselves, a heart rate (HR) monitor and an oximeter (on to the great toe of the non-dominant leg). Subjects will then undertake their allocated intervention as outline above. During this time HR and oxygen saturation

(OS) will be monitored. On completion, blood samples will be taken from both cannulae. Subjects will then be asked to sit and rest for 1 hour.

Next you will be asked lay in a supine (on your back) position, and provide a muscle sample obtained from the lateral side of the gastrocnemius (calf), which will be obtained by an experienced medical practitioner from MKGH. In addition to muscle samples, blood will also be extracted from both cannulae at this point. A tourniquet will then placed upon your non-dominant leg and inflated to 100 mmHg above resting systolic pressure for 45 minutes. During this time your HR and OS will be measured. In addition to these, your thermal sensation (how hot/cold you feel) and pain (via a visual analogue pain scale) will be taken in consideration to your whole body and subjected leg. Once the elected time has elapsed, the tourniquet and oximeter will be removed and another muscle and blood sample will be obtained 15 min post removal of the tourniquet from the same regions as previously stated.

Subjects will be required to return to have further blood and muscle samples taken 2 hours post removal of the tourniquet. Upon completion HR monitor will be removed.

What are the possible risks of taking part in the study?

There will be a trained first aider in the immediate vicinity throughout all testing. During the muscle biopsy and blood sampling there is a slight risk of infection and you may experience a degree of discomfort. However, the risk of infection will be kept to a minimum through use of a designated clinical area and performed using sterile techniques. The level of discomfort during blood sampling will be minimised through the use of a trained phlebotomist (individual who takes blood samples) and during the muscle biopsy an orthopaedic surgeon will be performing the procedure.
There is a potential risk of altitude sickness when exposed to oxygen levels of 14.5%. This risk is minimum and all researchers involved are aware of the symptoms. During the experimental trial you may experience some discomfort from the tourniquet, although once removed, this will subside.

What if you decide you want to withdraw from the project?

If, at any stage you wish to leave the project, then you can without given explanation. There will be no disadvantage to yourself should you wish to withdraw.

What will happen to the data and information collected?

Everyone that takes part in the study will receive their own results for the tests that they complete for your own personal development and understanding. All information and results collected will be remain anonymous and held securely at the University of Bedfordshire and will only be accessible by the project team. Results of this project may be published, but any data included will in no way be linked to any specific participant. Your anonymity will be preserved.

What are the potential benefits of the study?

The present study will determine whether hypoxic and ischaemic interventions reduce the amount of tissue damage sustained after tourniquet application. Consequentially, if

successful, may reduce time needed for rehabilitation inducing reductions in cost for hospitals and clinics. In addition participants will obtain a full body composition profile (% fat and lean mass) worth £50.

Questions are always welcome and you should feel free to ask either myself, James Barrington, Dr Lee Taylor or an independent contact, Professor Angus Duncan at anytime. See details below for specific contact details.

If you are interested in taking part in the project and would like to receive more details about the studies please send an email to either:

James Barrington: 07734821427
Email: James.Barrington@beds.ac.uk

Dr Lee Taylor
Email:Lee.Taylor@beds.ac.uk

Department of Sport and Exercise Sciences,
University of Bedfordshire
Bedford Campus,
Polhill Avenue, Bedford

Professor Angus Duncan
Email:Angus.Duncan@beds.ac.uk

Appendix C

CONSENT FORM

TO BE COMPLETED BY PARTICIPANT

NAME:……(Participant)

I have read the Information Sheet concerning this project and understand what it is about. All my further questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:

- My participation in the project is entirely voluntary and I am free to withdraw from the project at any time without disadvantage or prejudice.
- I will be required to attend testing in the sport and exercise science laboratories on 2 separate occasions to complete the project.

- As part of the study I will have to:

  - Undergo body composition measurements through use of the bodpod.
  - Have blood pressure measured.
  - Provide a urine sample for testing pretesting.
  - Have a cunnula placed in both antecubital region (inside of the arm) and in the small saphenous vein (back of the calf).
  - Have heart rate monitored throughout testing.
  - Have oxygen saturation levels measured throughout testing.
  - Be exposed to one of the four following interventions:
    - Normoxic environment (20.9% O_2, 775 mmHg) at rest for 40 mins.
    - Hypoxic environment (14.5% O_2, 775 mmHg) at rest for 40 mins.
    - 4 cycles of 5 mins compression and 5 mins reperfusion from a tourniquet inflated to a 100 mmHg above resting systolic pressure placed on the thigh.
    - Control environment – seated in the laboratory at rest for 40 mins.
  - Undergo 45 mins of tourniquet compression inflated to a 100 mmHg above resting systolic pressure placed on the thigh.
  - Give an indication of pain and ratings of thermal sensation throughout testing.
  - Provide 10 blood samples and 3 skeletal muscle biopsies.

- I am aware of any risks that may be involved with the project.

- All information and data collected will be held securely at the University indefinitely. The results of the study may be published but my anonymity will be preserved.

Signed:…… (Participant) Date: ……

Appendix D

BLOOD ANALYSIS

Please read the following:

a. Are you suffering from any known active, serious infection?
b. Have you had jaundice within the previous year?
c. Have you ever had any form of hepatitis?
d. Have you any reason to think you may be HIV positive?
e. Have you ever been involved in intravenous drug use?
f. Are you a haemophiliac?
g. Is there any other reason you are aware of why taking blood might be hazardous to your health?
h. Is there any other reason you are aware of why taking your blood might be hazardous to the health of the technician?

Can you answer **Yes** to any of questions a-g? Please tick your response in the box below:

Yes ☐ No ☐

Small samples of your blood (from finger or earlobe) will be taken in the manner outlined to you by the qualified laboratory technician. All relevant safety procedures will be strictly adhered to during all testing procedures (as specified in the Risk Assessment document available for inspection in the laboratory).

I declare that this information is correct, and is for the sole purpose of giving the tester guidance as to my suitability for the test.

Name ……

Signed ……

Date ……

If there is any change in the circumstances outlined above, it is your responsibility to tell the person administering the test immediately.

The completed Medical Questionnaire (Par Q) and this Blood Sampling Form will be held in a locked filing cabinet in the Department of Sport and Exercise Science

laboratories at the University for a period of one-three years. After that time all documentation will be destroyed by shredding.

Appendix E

General pre-test medical questionnaire

To be completed by all subjects before participating in practical sessions.

Name: …….

Age:…… Gender: M / F

1 Are you in good health? Yes / No
If no, please explain:

2 Are you pregnant or have you given birth in the last 6 months? Yes / No

3 How would you describe your present level of moderate activity?
< once per month
once per month
2-3 times per week
4-5 times per week
> 5 times per week

4 Have you suffered from a serious illness or accident? Yes / No
If yes, please give particulars:

5 Are you recovering from an illness or operation? Yes / No
If yes, please give particulars:

6 Do you suffer, or have you ever suffered from:
Respiratory conditions (asthma, bronchitis, tuberculosis, other)? Yes / No
Diabetes? Yes / No
Epilepsy? Yes / No
High blood pressure? Yes / No
Heart conditions or circulation problems:
(angina, high blood pressure, varicose vein, aneurysm, embolism, heart attack, other)?
Do you have chest pains at any time? Yes / No
Do you suffer from fainting/blackouts/dizziness? Yes / No
Is there any history of heart disease in your family? Yes / No

7 Are you currently taking medication ? Yes / No
If yes, please give particulars:

8 Are you currently attending your GP for any condition or have you consulted your doctor in the last three months? If yes, please give particulars: Yes / No

9 Have you had to consult your doctor, or had hospital treatment within the last six months? Yes / No

10 Have you, or are you presently taking part in any other laboratory experiment? Yes / No

PLEASE READ THE FOLLOWING CAREFULLY

Persons will be considered unfit to do the experimental exercise task if they:

have a fever, suffer from fainting spells or dizziness;
have suspended training due to a joint or muscle injury;
have a known history of medical disorders, i.e. high blood pressure, heart or lung disease;
have had hyper/hypothermia, heat exhaustion, or any other heat or cold disorder;
have anaphylactic shock symptoms to needles, probes or other medical-type equipment.
have chronic or acute symptoms of gastrointestinal bacterial infections (e.g. Dysentery, Salmonella)
have a history of infectious diseases (e.g. HIV, Hepatitis B); and, if appropriate to the study design, have a known history of rectal bleeding, anal fissures, haemorrhoids, or any other condition of the rectum;

DECLARATION

I hereby volunteer to be a subject in experiments/investigations during the period of 20___**.**

My replies to the above questions are correct to the best of my belief and I understand that they will be treated with the strictest confidence. The experimenter has explained to my satisfaction the purpose of the experiment and possible risks involved.

I understand that I may withdraw from the experiment at any time and that I am under no obligation to give reasons for withdrawal or to attend again for experimentation.

Furthermore, if I am a student, I am aware that taking part or not taking part in this experiment, will neither be detrimental to, or further my position as a student.

I undertake to obey the laboratory/study regulations and the instructions of the experimenter regarding safety, subject only to my right to withdraw declared above.

Name of subject (please print)

_____

Signature of Subject _____ Date:

Name of Experimenter (please print)_____

Signature of Experimenter _____ Date:

Appendix F

Muscle Biopsy Procedure

The biopsy site should then be cleaned using an alcohol spray or wipe.

The skin, adipose tissue and skeletal muscle fascia should then be anaesthetised using 5cm^3 of 1% lidocaine being injected into the biopsy site in 2.5 cm^3 doses at 45° proximal and 45° distal to the biopsy site respectively. Allow 3-5 min for anaesthetic to take effect.

Apply chlorhexodine to skin in preparation to biopsy site and surrounding leg.

A #11 scalpel should then be used to make a 4-5mm longitudinal incision.

The "non biopsy hand" should then grip muscle on superior side of the leg. The biopsy needle should be inserted into incision at an angle perpendicular to skin surface. Once resistance has been met, flatten the angle of the needle to 45°. Then press the biopsy gun button and remove needle quickly.

Allow assistant to remove tissue from needle (to be snap frozen in liquid nitrogen) whilst applying pressure to the wound with sterile gauze.

Any subsequent passes (repeat of above) should be lateral to the previous pass.

Following the last pass, firm pressure should be applied for up to 5 mins (or until bleeding stops).

Apply Steristrip (2 cm length) across the incision and then a dressing. Clean leg before participant leaves the room.

Once participant leaves room, spray bed clean and wipe down.